Vol. 71, No. 4 December 1973

THE JOURNAL OF HYGIENE

(*Founded in 1901 by G. H. F. Nuttall, F.R.S.*)

CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London NW1 2DB

American Branch: 32 East 57th Street, New York, N.Y.10022

1973 *subscription per volume* £11.50 *net* (*US* $38.00) (4 parts)

Single parts £4.00 *net* (*US* $13.00)

The Journal of Hygiene

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The Journal of Hygiene is published quarterly.

Single parts £4.00 net (US $13.00 in the U.S.A. and Canada) plus postage.

Four parts form a volume. The 1973 subscription price of a volume (which includes postage) is £11.50 net (US $38.00 in the U.S.A. and Canada).

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The Journal of Hygiene 1974

In 1974 the Journal will be published as two volumes of three parts each and the annual extent will be increased by 128 pages to 960 pages. The subscription price of the Journal for Volumes 72 and 73 (1974) will be £8 per volume, i.e. £16 per year.

J. Hyg., Camb. (1973), **71**, 633
Printed in Great Britain

Particle concentrations in patient rooms with various types of ventilation

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(*Received* 22 *January* 1973)

SUMMARY

Particle concentration in patient rooms at various ventilation rates and with differing methods of air supply has been investigated experimentally in a specially built test room. The size of the test room corresponded to an ordinary bedroom for two patients. Air was supplied isothermally to this test room, either through induction units or through a perforated ceiling, and the number of air changes per hour varied from 1 to 16. When the air was supplied by means of induction units, tests with different airflow patterns in the room were also carried out. The principle of the measurements was to estimate the decrease in particle concentration at certain points when the initial concentration was uniform all over the room and no generation took place during the measuring period. Talc powder was used as test dust, and the particle concentration was measured at up to six points in the room with a particle counter.

The measurements show that air-supply method and airflow pattern affect the particle concentration, but that the differences in concentration are small compared with those obtained when the ventilation rate is varied. However, the results should not be directly applied to systems with particle generation.

INTRODUCTION

The design of ventilation systems in hospitals has been treated by several authors from the point of view of limiting the bacterial air contamination (Charnley & Eftekhar, 1969; Galson & Goddard, 1968; Goddard, 1966; Lidwell, Richards & Polakoff, 1967). In this connexion the influence of the ventilation rate and of the types of air diffusers selected, and their location, has been discussed. It has also been shown that bacteria in the air are to a great extent carried by particles (Goddard, 1966; Noble, Lidwell & Kingston, 1963), and therefore the risk of infection can be minimized by limiting the concentration of airborne particles.

The present experimental investigation is a continuation of a previous paper (Allander & Faxvall, 1971), in which results from measurements of the particle concentration in patient rooms were presented, together with a theoretical analysis

of how the particle concentration varies with the ventilation rate and the particle size. It was found in this previous investigation that, especially for particles $\gtrsim 5\mu$m., the particle concentration may increase considerably if the air supply system gives rise to an airflow pattern with vortices. As particles of this size can be expected to carry bacteria, it was regarded as necessary to continue with further experimental investigations.

The investigation presented below is a first step in an attempt to obtain an answer to the question of whether gravity and particle diffusion affect the transport of airborne particles in ventilated rooms, or whether these factors are negligible. The experiments were carried out at the laboratories of AB Svenska Fläktfabriken in Jönköping, whereas the previous experiments were carried out in patient rooms at two hospitals in the Stockholm area – one with modern ventilation and the other without mechanical ventilation. As the measurements were made in a laboratory, it was possible to control the generation of particles and to vary the ventilation rate within wide limits. Moreover, such differing methods of air supply as through induction units and through perforated ceilings could be tested in one and the same room.

As the tests were carried out in a system without particle generation, the results cannot be directly applied to systems where particles are continuously generated without further investigation.

TEST ROOM AND MEASURING EQUIPMENT

The experiments were carried out in a test room 6 m. long, 3·6 m. wide and 2·7 m. high. A room of this size corresponds to an ordinary patient room for two patients. The walls of the room were made of double plates of Masonite with an insulating layer of Rockwool 50 mm. thick. To keep the wall temperature constant, air at a controlled temperature was circulated around the test room.

When the air was supplied with induction units three SANIVENT units, type RBAA-09 (AB Svenska Fläktfabriken), were used. This induction unit is intended for local heating of pretreated air, and it has rotatable grilles through which the primary airflow can be directed. The unit has no inlet for secondary airflow, as it is mainly intended for use in hospitals, where secondary air through induction units should be avoided for hygienic reasons.

When supplying the air through perforated ceilings it is very difficult to keep the airflow equally distributed over the whole ceiling. A ceiling was therefore built with several layers, i.e. the airflow had to pass an absolute filter, a perforated steel plate with 3 mm. holes 5 mm. apart, a grille with a thickness of 20 mm. and square holes 20×20 mm., and finally a perforated steel plate with 3 mm. holes 25 mm. apart.

During the test with the induction units the exhaust air was vented through three KGE registers (AB Svenska Fläktfabriken), which during the first part of the investigation were mounted in the ceiling near the inner wall and later in the inner wall near the ceiling. During the experiments with the perforated ceiling the room air was exhausted through slots near the floor in each of the intermediate

walls. In both cases the temperature and the humidity of the supply air were controlled. To keep down the particle concentration in the room the supply air was cleaned by means of an absolute filter.

The particle concentration was measured by means of a ROYCO 220 particle counter, with additional equipment for automatic data logging (Department of Heat Technology, Royal Institute of Technology). With this instrument the number of particles per 2·8 l. (0·1 ft.^{3}) is measured within six size ranges, $>0{\cdot}3$, $>0{\cdot}5$, >1, >2, >5 and $>10\,\mu$m. The number of particles in each range is printed out about 50 times/hr. (The time between the measuring series is 78 sec.) For further computer analyses the measured values can be transferred to a tape punch.

An artificial test dust with known size distribution was regarded as most suitable for the experiments. In view of the results from the previous investigation for particles $>5\,\mu$m. and $>10\,\mu$m., the test dust should contain a considerable fraction of particles in the range, say, 5–20 μm. It was found that talc powder, Microdol-1 (A/S Norwegian Talc), has about the desired size distribution. This test dust was supplied to the room by means of compressed air. It should be observed that the talc particles are far from spherical and that the particle diameters measured with the ROYCO counter may differ from the aerodynamic diameters of the particles.

EXPERIMENTAL PROCEDURE

When it is important to keep down the particle concentration in a room, a ventilation system with air supply through a perforated ceiling and air exhaust near the floor should be almost the ideal solution. But a perforated ceiling is difficult to keep clean and this solution is therefore not very useful in practice. A certain measure of the ability of a ventilation system to carry away particles from a room can, however, be obtained by making a comparison with a system with a perforated ceiling. The first part of the present investigation is devoted to a comparison of the particle concentration in the test room when it is ventilated in a conventional way by means of induction units, with the concentration when the air is supplied through a perforated ceiling. During the second part of the investigation the airflow pattern in the room was varied when supplying the air with induction units. Different airflow patterns were produced by placing a screen in the ceiling, or by supplying the air with a relatively low discharge velocity.

All experiments started with the supply of the test dust (the talc powder) to the room air. The dust was distributed uniformly over the whole volume of the test room by means of a fan placed in the room. The time required for adding and distributing the test dust was 5 min. (During this time the ventilation system was in operation.) The decay of the particle concentration was then measured for the particle sizes $>0{\cdot}3$, $>0{\cdot}5$, >1, >2, >5 and $>10\,\mu$m. As the measured values for the range $>0{\cdot}3\,\mu$m. are uncertain, those values were cancelled.

For the various particle sizes the concentration values measured were plotted in a semilogarithmic diagram. If complete mixing of the dust occurs in the test room, straight lines are obtained in these diagrams, as the decay then follows an

exponential function. Accordingly the particle concentration C_t at the time t can be written

$$C_t = C_0 \exp\left\{-\frac{q}{V}t\right\},$$

where q is the ventilation airflow and V the volume of the room. The decay constant for the particle concentration decrease is then

$$k = q/V = n,$$

where n is the number of air changes per time unit.

By expressing the results in terms of the decay constant instead of the absolute value of the particle concentration after a certain time, the results are independent of the original particle concentration. This is important as there are experimental difficulties in keeping the original concentration constant. It is true that a specific quantity of dust was added at each experiment (0·3 or 0·5 g.), but part of the test dust is lost by sedimentation during the mixing period. As it is difficult to keep the mixing and distributing conditions constant, improved accuracy is obtained by measuring the decay constants.

All the tests were carried out under the following conditions: supply air temperature, 20° C., wall temperature, 20° C., room temperature, 20° C., relative humidity, 40–50 %.

During the first part of the investigation, i.e. that dealing with the direct comparison between the induction units and the perforated ceiling, the particle concentration was measured at six points in the room (in a plane through the centre of the room, parallel with the intermediate walls). The positions of the measuring points are shown in Fig. 1 (airflow pattern I), where the notation for these points is also given. The tests were carried out at 1, 2, 4, 8 and 16 air changes/hr. One series of measurements was carried out with the ventilation system shut off ($n = 0$).

During the second part of the investigation the particle concentration was measured for two other airflow patterns in the room when $n = 2$, 4 and 8 air changes/hr.

In these cases measurements were only made at points C1 and C2, or C1, C2 and I2. The three airflow patterns (Fig. 1) can be described as follows:

(I) Airflow pattern in which the supply air flows upwards along the window wall, follows the ceiling to the inner wall, and then flows down the inner wall to the floor, where it turns back into the room. This corresponds to a 'normal' airflow pattern in the room.

(II) Airflow pattern in which the supply air flows upwards along the window wall, follows the ceiling for about 3 m. and then flows downwards into the room. Near the floor, part of the air flows towards the window wall and part of it towards the inner wall. To effect this airflow pattern a screen was put up in the ceiling, 3 m. from the inner wall.

(III) Airflow pattern in which the supply air flows upwards along the window wall, turns at the ceiling and flows downwards. Near the floor, part of the air flows towards the inner wall and part of it is ejected upwards by the supply air.

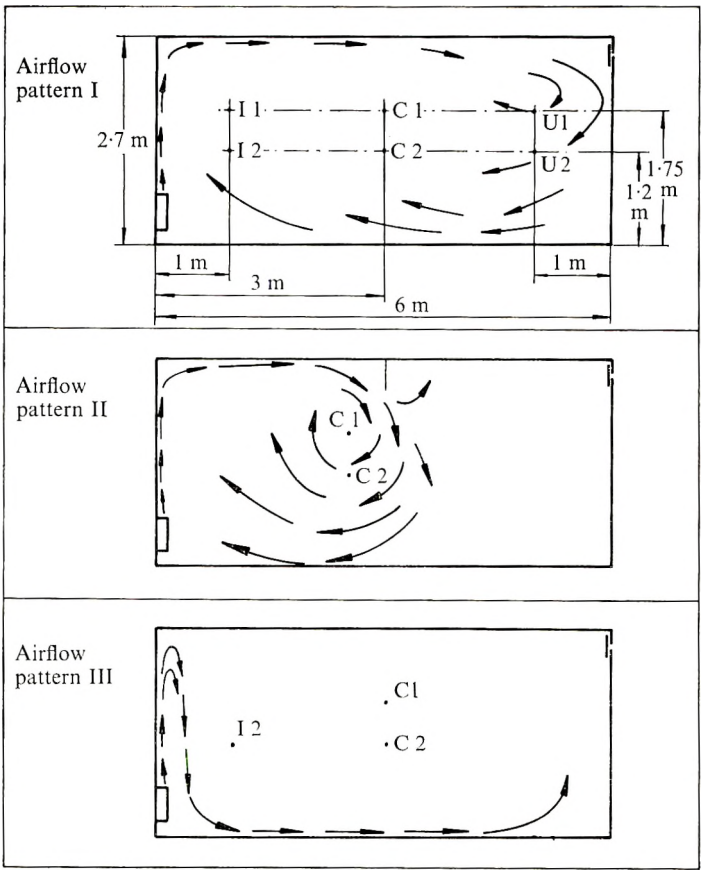

Fig. 1. Three airflow patterns investigated, with air supply through induction units. Also shown are the positions and notations of the measuring points.

To effect the low discharge velocity required for this flow pattern, the rotatable grilles of the induction units were replaced by perforated plates with suitable perforation.

RESULTS

The decay constants for the comparative investigation of induction units and perforated ceiling and for the investigation of different airflow patterns were obtained by fitting straight lines to the measured particle concentrations in semi-logarithmic diagrams. The decay follows an exponential function quite well for the various measuring points and ventilation rates.

In Table 1 the decay constant measurements are summarized. It is clear that the particle concentration decays with almost the same rate when the air is supplied with induction units and perforated ceiling. On the average, only a slightly faster decay is obtained for the perforated ceiling, due to increased sedimentation.

A comparison of the measured values for the normal airflow pattern and the case when the supply air is forced downwards in the middle of the room shows, as could be expected, that the particle concentration decays more slowly in the latter case

Table 1. *Comparison of the decay constant mean values when the air is supplied through induction units (k_i) and a perforated ceiling (k_p)*

(The airflow patterns I, II and III are shown in Fig. 1.)

Ratio of decay constants	Particle size (μm.)	k_i/k_p				
		n = 1 hr.$^{-1}$	n = 2 hr.$^{-1}$	n = 4 hr.$^{-1}$	n = 8 hr.$^{-1}$	n = 16 hr.$^{-1}$
Induction I/perf. ceiling	> 0·5	0·98	0·96	0·89	0·94	0·88
	> 1	1·00	1·00	0·94	0·98	0·89
	> 2	0·84	0·83	0·91	0·93	0·88
	> 5	0·92	0·96	0·97	0·99	0·93
	> 10	0·92	0·98	1·04	1·11	1·05
Induction II/perf. ceiling	> 0·5	—	0·80	0·65	0·81	—
	> 1	—	0·83	0·71	0·84	—
	> 2	—	0·84	0·78	0·86	—
	> 5	—	0·96	0·82	0·88	—
	> 10	—	0·82	0·80	0·86	—
Induction III/perf. ceiling	> 0·5	—	0·91	0·70	0·77	—
	> 1	—	0·96	0·70	0·80	—
	> 2	—	1·04	0·86	0·82	—
	> 5	—	1·09	0·87	0·82	—
	> 10	—	0·97	0·87	0·77	—

(at points C1 and C2). The difference is most evident for small particles. It should be observed that the measuring points C1 and C2 were moved about 0·5 m. to the left when the airflow pattern II was investigated. The point C1 was then very close to the vortex centre.

For airflow pattern III the particle concentration (at points C1, C2 and I2) decays more slowly than for a normal flow pattern in the room. Here also the difference is most evident for small particles.

When the air is supplied through induction units (airflow pattern I) the particle concentration decreases with almost the same rate for all the measuring points. The coefficient of variation is less than 0·05 in this case. When the air is supplied through a perforated ceiling the measured decay constants have a considerable spread particularly when the ventilation rate is low, i.e. the coefficient of variation is about 0·20. The reason for this is presumably the reduction in turbulence which becomes insufficient to maintain mixing within the room, and the average velocity at which the air is supplied through the ceiling, no more than 0·001 m./sec. at n = 1 hr.$^{-1}$, is too low to produce so-called piston ventilation. A temperature difference between walls and air of only 0·01° C. will generate a convectional velocity near to the wall of as much as 0·013 m./sec.*

The mean values of the measured decay constants for the case $n = 0$ and for airflow pattern I are plotted in Fig. 2. The results show that the rate of clearance falls off for both large and small particle sizes, especially the latter, when the

* The maximum velocity produced near a vertical wall $v_{max.} = c\sqrt{(H\Delta\theta)}$, where c is a constant, typically about 0·08, H is the height of the wall and $\Delta\theta$ the temperature difference (Eckert & Drake, 1959). For H = 2·7 m. and $\Delta\theta$ = 0·01° C., $v_{max.}$ = 0·013.

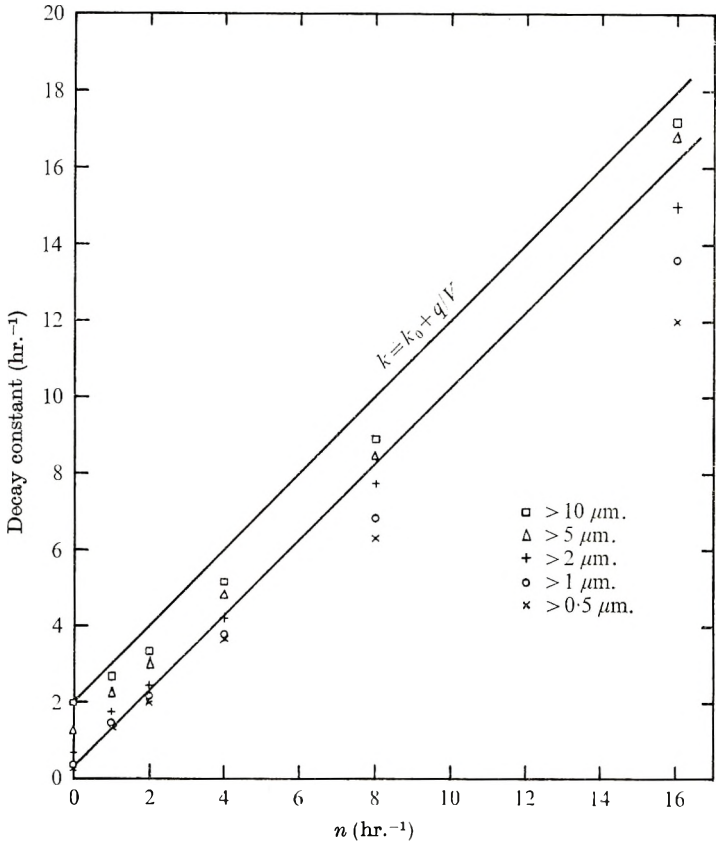

Fig. 2. Decay constants for various particle sizes and ventilation rates when the air is supplied through induction units (airflow pattern I). The parallel lines for the particle sizes $>0{\cdot}5$ and >10 μm. are derived from the settling rates, $n = 0$, and the ventilation supply rates.

ventilation supply is increased. The effect at small particle sizes is probably due to incomplete mixing in the room, i.e. the effective ventilation flow is less than the real flow.

DISCUSSION

The experiments in the laboratory test room concerning the decay of the concentration of an artificial test dust lead to certain rules for the design of ventilation systems for patient rooms from the point of view of bacterial contamination.

According to the test results the decay constant for the particle concentration is directly proportional to the ventilation rate for the particle sizes of interest. Thus the ventilation rate should be chosen as high as possible with respect to draught problems in the room.

The comparison between induction units and perforated ceilings shows that somewhat lower particle concentrations should be obtained if the air is supplied by means of a perforated ceiling instead of an induction unit, owing to increased sedimentation. But the improvement is small, and it is difficult to find any

practical method of supplying the air which gives substantially lower particle concentrations than those obtained by means of induction units, provided that these units are carefully adjusted and do not give rise to vortices.

As was found in the previous investigation (Allander & Faxvall, 1971), the comparison between the three airflow patterns shows that the particle concentration increases if the airflow pattern contains vortices or areas with stagnant air. The difference, however, is not as evident as might have been expected from the previous investigation.

REFERENCES

ALLANDER, C. & FAXVALL, S. (1971). Theoretical and practical investigation of particle concentrations in patient rooms with various types of ventilation. *VVS* **42** (9), 61 (in Swedish).

CHARNLEY, J. & EFTEKHAR, N. (1969). Postoperative infection in total prosthetic replacement arthroplasty of the hip-joint. *British Journal of Surgery* **56**, 642.

ECKERT, E. R. G. & DRAKE, R. M. JR (1959). *Heat amd Mass Transfer*, pp. 311–14. New York: McGraw-Hill.

GALSON, E. & GODDARD, K. R. (1968). Hospital air conditioning and sepsis control. *ASHRAE Journal*, July 1968, p. 33.

GODDARD, K. R. (1966). Hospital ventilation system design. *ASHRAE Journal*, October 1966, p. 46.

LIDWELL, O. M., RICHARDS, I. D. G. & POLAKOFF, S. (1967). Comparison of three ventilating systems in an operating room. *Journal of Hygiene* **65**, 193.

NOBLE, W. C., LIDWELL, O. M. & KINGSTON, D. (1963). The size distribution of airborne particles carrying micro-organisms. *Journal of Hygiene* **61**, 385.

J. Hyg., Camb. (1973), **71**, 641
Printed in Great Britain

The efficacy of live and inactivated vaccines of Hong Kong influenza virus in an industrial community

A report to the Medical Research Council Committee on Influenza and other respiratory virus vaccines.*

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(*Received* 26 *February* 1973)

SUMMARY

Intranasal vaccines of inactivated or living attentuated A2/Hong Kong influenza viruses were compared for clinical acceptability, serological effects and protective efficiency against natural epidemic influenza in a large industrial and clerical population.

Neither vaccine resulted in any significant untoward side-effects. The serum haemagglutination-inhibiting (HI) antibody response within 1 month of vaccination was similar with both vaccines; approximately 50% of those with little or no pre-vaccination antibody developed 4-fold or greater rises in titre. The effect of the antigenic potency of the vaccines and the prior immunological experience of the population is discussed. Volunteers given live vaccine showed a 2·2-fold lower incidence of clinical influenza than those given killed vaccine in a natural epidemic 16 months after vaccination.

INTRODUCTION

In previous small-scale clinical trials (e.g. Beare, Hobson, Reed & Tyrrell, 1968) living influenza virus vaccines given intranasally to adult volunteers induced an immune response, especially in terms of circulating antibody against the viral haemagglutinin (HA) antigen, which conferred protection against intranasal challenge infection with a homologous virus strain 2–4 weeks after vaccination. The live vaccines were easily administered by nasal drops or spray, appeared to evoke few or no clinical signs or symptoms of infection, and the amount and duration of virus excretion was low.

* Members: Prof. Sir Charles H. Stuart-Harris (Chairman), Prof. G. Belyavin, Dr J. T. Boyd, Prof. G. W. A. Dick, Prof. Sir Austin Bradford Hill, Dr F. Himmelweit, Dr D. Hobson, Dr W. W. Holland, Sir James Howie, Dr F. O. MacCallum, Dr H. G. Pereira, Dr F. T. Perkins, Dr T. M. Pollock, Dr A. T. Roden, Dr D. A. J. Tyrrell and Dr A. S. Beare (Secretary).

The purpose of the present trial was to determine (*a*) whether live intranasal vaccines are equally acceptable under field conditions in large industrial and clerical populations, (*b*) whether killed intranasal vaccine would be equally immunogenic but less reactogenic, as suggested by the studies of Waldman, Small & Rowe (1970) and (*c*) whether significant protection could be demonstrated against any natural influenza epidemic occurring in the community in the subsequent two years.

MATERIALS AND METHODS

In November 1970, 3 schedules of vaccination were compared in randomly chosen groups of volunteers in the headquarters staff of the Midland Region of the Central Electricity Generating Board in Solihull, Warwickshire. Group 1 received two intranasal doses, at fortnightly intervals, of a live attentuated strain of A2/Hong Kong virus. Group 2 were given placebo material in the first session, to facilitate the investigation of clinical responses to the live vaccine, and a single dose of live vaccine two weeks later, at the time Group 1 were receiving their second dose. Group 3 were given two intranasal doses at fortnightly intervals of a killed vaccine containing the recombinant virus X31 which is antigenically identical with A2/Hong Kong (Kilbourne *et al.* 1971). Details of these vaccines are given below. A total of 450 H.Q. staff were vaccinated; a detailed questionnaire on clinical reactions to the vaccine was given to each volunteer, and blood samples were obtained before and after vaccination from 147 of them.

An extended but somewhat simplified trial of the same vaccines was also conducted in November 1970 in the generating stations of C.E.G.B. Midland region. The main purpose was to allow larger-scale investigations of the protective effect of the vaccines in the event of an influenza epidemic, and no studies were made of the serological effect of the vaccines or of clinical side-effects to them; 1836 volunteers from a total working population of 15,592 were randomly allocated to receive either a single dose of live Hong Kong vaccine or a single dose of killed X31 vaccine. All sickness absence due to respiratory infection in each vaccinated group, and in the total H.Q. and station population, was evaluated over the periods November to March (pay weeks 35–52) of 1970–1 and 1971–2.

All the vaccines in the present trial were made and generously given by Evans Medical Limited, Speke, Liverpool. The live vaccine was prepared from a seed pool of an inhibitor-resistant strain A2/HK/1/68/EG.972 which had previously undergone serial allantoic passages in chick embryos in the presence of horse serum prior to terminal dilution purification in RIF-free eggs. A single allantoic fluid pool was made in RIF-free eggs, purified by differential centrifugation and freeze-dried in 1 ml. amounts (ten doses) in ampoules in a freeze-drying medium routinely used in commercial measles vaccine manufacture. The vaccine was freshly reconstituted in distilled water just before use; the virus titre of each ampoule was $10^{7\cdot2}$ EID_{50} (egg infective doses) per ml. The placebo vaccine consisted of freeze-drying medium only, dispensed in ten-dose ampoules, and was identical with the live vaccine except for a 'blind' letter-coding. The killed vaccine was an aqueous suspension of X31 virus, inactivated with 1/1000 β-propiolactone; each 0·1 ml.

Table 1. *The effect of intranasal Hong Kong influenza virus vaccines on homologous HI antibody titres in serum*

		Vaccine group 1	2	3
Total of volunteers tested		53	43	51
No. without pre-vaccine antibody (< 6)		18 (34 %)	15 (35 %)	16 (31 %)
No. with ⩾ fourfold rise in titre		12 (23 %)	1 (2·3 %)	12 (23·5 %)
No. with twofold rise in titre		7 (13 %)	2 (4·6 %)	14 (27 %)
No. with ⩾ twofold *fall* in titre (estimate of technical error in above)		0	2	0
No. of ⩾ fourfold rises in persons of different pre-vaccine titres (% of total at each initial level)	< 6	6 (34 %)	1 (8 %)	6 (36 %)
	6	5 (80 %)	0	2 (66 %)
	12	1 (12 %)	0	3 (34 %)
	24	0	0	1 (16 %)
	48	0	0	0
G.M.T. of whole group	Pre-vaccine	9·3	9·1	11·5
	Post-vaccine	17·8	9·8	25·1

Vaccination schedules

	1st dose	2nd dose
Group 1	Live HK	Live HK
Group 2	Saline	Live HK
Group 3	Inactivated HK	Inactivated HK

Interval between doses = 2 weeks.
2nd serum 2 weeks after 2nd dose.

dose contained 1500 HAU (approx. ten times more HA units than the dose of live vaccine). Each type of vaccine was given in a 0·1 ml. dose, placed dropwise high into the volunteer's nostrils from disposable plastic syringes fitted with a 3-in. 18-gauge metal cannula. The patient was instructed to keep his head tilted back, and sniff up the inoculum for one minute after instillation, and was told not to blow his nose for one hour. Separate operators and equipment in well-segregated areas of the clinic were used for each vaccine.

Serum samples were stored at $-20°$ C., and titrated in pairs for HI antibody, after treatment with cholera filtrate to destroy non-specific virus inhibitors. The homologous live vaccine strain of HK virus was used throughout; 4 HA units of virus were incubated with serum dilutions for 1 hr. at room temperature before adding a 1 % suspension of fowl red cells. The general performance of the test was as for the W.H.O. plastic plate method (W.H.O. Report, 1953).

RESULTS

Serological responses to vaccination

Details of the effect of each vaccine on serum HI antibody titres are shown in Table 1, from which it is apparent that almost all increases in titre were in those initially without detectable antibody or with pre-vaccine titres of 24 or less. Two doses of live or killed vaccine were equally effective, inducing fourfold or greater

Table 2. *The clinical effects of intranasal vaccines of Hong Kong influenza virus*

Clinical symptoms notified	No. of volunteers with symptoms within 7 days of first dose: Group 1, live vaccine	Group 2, placebo	Group 3, inactivated vaccine
Sneezing	28	21	21
Stuffy or running nose	31	39	38
Sore throat or cough	30	19	21
Headache	23	24	22
General malaise, or aching limbs	19	25	21
Off work, with any of above symptoms	3	3	4
Total no. of volunteers with one or more symptoms	54	58	57
Total no. in each group	107	107	124

Completed questionnaires were returned by 75% of those vaccinated.

rises in titre in 23% and 23·5% of volunteers respectively. A single dose of live vaccine appeared to have no significant effect on HI titres, but the post-vaccination HI test in this group was only two weeks after their single dose of vaccine whereas, in the other two groups, the interval between the first dose of vaccine and the post-vaccination sample was four weeks. Hence, if the immune response to vaccine is mainly attributable to the first dose, as in our previous trials (Beare *et al.* 1969), rises in titre would be more likely to have occurred in Groups 1 and 3 than in Group 2, at the time of sampling.

Clinical effects of vaccination

Questionnaires were returned by 338 (75%) of H.Q. staff after their first dose of vaccine. The analysis of responses (Table 2) shows that half the volunteers reported one or more symptoms, but that neither of the vaccines gave an enhanced or different pattern of reactions compared with the placebo material. Thus the physical or subjective effects of intranasal inoculation seem of greater importance than the content of the inocula. Similarly, after the second dose of vaccine there was no significant difference in the frequency of symptoms in those in Group 1 who had already had a previous dose of live vaccine and those in Group 2 receiving vaccine for the first time.

The protective effects of vaccination

The sickness absence of all volunteers and non-vaccinated personnel on all the stations in the trial was recorded by computer from the diagnosis given in the medical certificate from the patient's family doctor.

The number of illnesses attributed to influenza, coryza and bronchitis was calculated separately. Since many respiratory illnesses, even mild attacks of influenza, might cause a loss of less than 3 working days, computer data on short 'uncertified illness' were also examined. Data for the whole study population, including H.Q.

Table 3. *Differences in the incidence of respiratory illness in vaccinated and unvaccinated personnel*

(Data for categories in which there were no significant differences at any time in either winter between any of the groups have been omitted. Group L, live HK vaccine, 910 persons. Group K, killed HK vaccine, 926 persons. Group U, no vaccine, 13,756 persons.)

Data on time off work in each pay-week period	Sole category of difference in year of observation over period 1 November–1 April					
	1970–1, Bronchitis			1971–2, Influenza		
	L	K	U	L	K	U
Weeks 35–9						
No. ill	8	6	134	7	7	68
% ill	0·8	0·6	1·1	0·8	0·8	0·5
Days absent/person	7·5	6·5	12·3	5·1	8·0	6·4
Weeks 40–3						
No. ill	14	12	243	8	10	123
% ill	1·5	1·3	1·9	0·9	1·1	0·9
Days absent/person	7·0	11·1	11·0	6·9	6·1	6·7
Weeks 44–7						
No. ill	8	6	183	8	21	268
% ill	0·8	0·6	1·4	0·9	2·3	1·9
Days absent/person	6·7	9·5	10·8	7·7	6·2	7·0
Weeks 48–52						
No. ill	3	7	149	9	15	195
% ill	0·3	0·7	1·2	1·0	1·6	1·4
Days absent/person	5·7	6·4	9·7	10·3	5·9	6·5

staff, were reviewed collectively simply as live vaccine versus killed vaccine versus unvaccinated personnel. The errors inherent in comparing those who volunteer for vaccination with those who do not are well recognized; however, since the nature of the trial precluded a group of volunteers given only placebo, it was felt worthwhile to include this extra statistic.

Data for 1970–1

In the first winter after vaccination, Hong Kong virus isolations were reported only very sporadically by the Public Health Laboratory Service in various parts of the country, and there were no frank epidemics. The computer data showed a constant low incidence of 'influenza', coryza and uncertified illness in all groups with no significant differences between them. On the other hand, illnesses certified as 'bronchitis' (see Table 3) were significantly less in each of the two vaccinated groups than in unvaccinated persons in pay weeks 44–7 ($\chi^2 = 5{\cdot}5$, $P < 0{\cdot}02$) and weeks 48–52 ($\chi^2 = 6{\cdot}8$, $P < 0{\cdot}02$). The differences between those given live and those given killed vaccine were not significant.

Data for 1971–2

There was a sharp increase in HK virus influenza throughout England early in December 1971, which reached a peak late in February 1972, and did not disappear

until mid-April 1972. Although no virological confirmatory tests were made in the C.E.G.B. population, it is reasonable to assume that the increase in illnesses certified as influenza in weeks 44–7 (see Table 3) were part of the HK epidemic in the general local population. In this period 268 (1·9 %) of the unvaccinated and 21 (2·3 %) of the group given killed vaccine had an influenzal illness as compared with only 8 (0·9 %) of those given live vaccines; these differences are statistically significant overall ($\chi^2 = 5{\cdot}9$, $P < 0{\cdot}05$). The sickness rate was significantly less after live vaccine than either killed vaccine ($\chi^2 = 5{\cdot}7$, $P < 0{\cdot}02$) or no vaccine ($\chi^2 = 5{\cdot}4$, $P < 0{\cdot}02$) whereas there was no significant difference between killed vaccine and no vaccine. In this second winter, unlike 1970–1, the incidence of bronchitis was not apparently influenced by prior vaccination.

DISCUSSION

Before vaccination, 67 % of volunteers had already acquired serum HI antibody against HK virus. The effect of live vaccine, as in previous trials (Hobson, Curry, Beare & Ward-Gardner, 1972) was almost entirely to immunize the previously non-immune rather than to boost the titre of those who already had prior experience. The serological response was as good with the inactivated as with the live intranasal vaccine, as indicated above. It may be important that on this occasion the antigenic mass of the original inoculum was ten times greater in the inactivated than in the live vaccine. It is also possible that the apparent improvement over results in our previous trials is due to the prior experience of HK antigen of most of the present population, perhaps even in some to those with no detectable serum antibody just before inoculation. When similar inactivated intranasal HK vaccine was given in 1968, before there had been extensive natural spread of the virus, the serological response was much less than after parenteral HK vaccine (Hobson *et al.* 1970), and there was no protection against the first HK epidemic, which occurred 2–4 months after vaccination (Tyrrell, Buckland, Rubenstein & Sharpe, 1970). Similarly, the serological and protective effects of HK-killed intranasal vaccines recently reported by Waldman & Coggins (1972) were considerably better than in their earlier trials (Waldman *et al.* 1970) when HK virus was new and the population less primed.

The protective effect against influenza of the live vaccine was greater than that of killed intranasal vaccine, even though they had produced comparable serological responses shortly after administration. It is thus probable that live vaccine induced a higher and more prolonged degree of local immunity in the respiratory tract.

It is more difficult to explain the apparent effect of both vaccines in reducing the incidence of bronchitis in 1970–1. It is probable that, in periods when influenza viruses are only sporadically circulating, many short individual illnesses caused by these agents are designated as colds or bronchitis, chills etc. Influenza vaccines may thus appear to protect against apparently unrelated syndromes (e.g. Reports 1959; 1964). Conversely, in periods when influenza is known to be epidemic, there is a tendency to certify bronchitis and febrile colds as influenza. In small controlled trials it is obviously possible to minimize these errors by

obtaining virological proof of all infections, or by the substitution of a deliberate challenge infection for a natural epidemic. In field trials of the present nature more precise data than those described here are only likely to be obtained by greatly increasing the scale of the trial or by waiting for the emergence of a new virus serotype to which the majority of unvaccinated people would be highly susceptible.

We are extremely grateful to all the volunteers for their keen co-operation, to Miss J. Manuel, the Regional Nursing Officer, and her staff in the Medical Department of C.E.G.B. (Midlands Region) for their enthusiastic work in the planning and conduct of the trial and to members of C.E.G.B. Planning Division for computerizing sickness absence data. We are indebted to Dr D. Rubenstein and Dr S. E. Reed for their help in the vaccination sessions and to Professor Sir Charles Stuart-Harris, Dr D. A. J. Tyrrell and other members of the M.R.C. Committee for their helpful advice on the organization of the trial and in the preparation of this report.

REFERENCES

Beare, A. S., Hobson, D., Reed, S. E. & Tyrrell, D. A. J. (1968). A comparison of live and killed influenza virus vaccines. *Lancet* ii, 418.

Beare, A. S., Tyrrell, D. A. J., Hobson, D., Howells, C. H. L., Pereira, M. S., Pollock, T. M. & Tyler, L. E. (1969). Live influenza B vaccine in volunteers. *Journal of Hygiene* **67**, 1.

Hobson, D., Baker, F. A., Chivers, C. P., Reed, S. E. & Sharp, D. (1970). A comparison of monovalent Hong Kong influenza virus vaccine with vaccines containing only pre-1968 Asian strains in adult volunteers. *Journal of Hygiene* **68**, 369.

Hobson, D., Curry, R. L., Beare, A. S. & Ward-Gardner, A. (1972). The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *Journal of Hygiene* **70**, 767.

Kilbourne, E. D., Schulman, J. L., Schild, G. C., Schloer, G., Swanson, J. & Bucher, D. (1971). Correlated studies of a recombinant influenza virus vaccine. I. Derivation and characterization of virus and vaccine. *Journal of Infectious Diseases* **124**, 449.

Report (1959). Field trial of influenza virus vaccine in patients with chronic bronchitis during the winter of 1957–58. Report by the joint working party of the Medical Research Council Committee on influenza and other respiratory virus vaccines and of the Research Committee of the British Tuberculosis Association. *British Medical Journal* ii, 905.

Report (1964). Clinical trials of oil adjuvant influenza vaccines 1960–63. Report to the Medical Research Council by its Committee on Influenza and other respiratory virus vaccines. *British Medical Journal* **2**, 267.

Tyrrell, D. A. J., Buckland, R., Rubenstein, D. & Sharpe, D. M. (1970). Vaccination against Hong Kong influenza in Britain, 1968–69. *Journal of Hygiene* **68**, 359.

Waldman, R. H. & Coggins, W. J. (1972). Influenza immunization. Field trial on a University campus. *Journal of Infectious Diseases* **126**, 242.

Waldman, R. H., Small, P. A. Jr. & Rowe, D. S. (1970). *The secretory immunologic system* (Ed. D. H. Dayton, Jr., P. A. Small, Jr., R. M. Chanock, H. E. Kaufman and T. B. Tomasi, Jr. U.S. Dept. of Health, Education and Welfare. Public Health Service), p. 129.

World Health Organization (1953). Expert Committee on Influenza. *Technical Report Series of the World Health Organization.* No. 64.

J. Hyg., Camb. (1973), **71**, 649
Printed in Great Britain

Common colds in Antarctica

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(*Received* 28 *February* 1973)

INTRODUCTION

Recent studies have established that the occurrence of common cold epidemics amongst men in Antarctica shows a pattern similar to that seen in other isolated communities such as Tristan da Cunha (Cameron & Moore, 1968; Shibli, Gooch, Lewis & Tyrrell, 1971). Colds occur during relief periods when ships and aircraft call, and disappear during the months of winter isolation, but no statistical analysis of the incidence of such colds is available.

There has been evidence to suggest that during the Antarctic winter men gain increased resistance to colds, so that during the summer relief periods they suffer from fewer and milder colds than do newcomers (Taylor, 1960; Hedblom, 1961). This resistance then appears to be lost when they return to less isolated communities, and their colds then tend to be more severe than those which they experienced while still in Antarctica (Cameron & Moore, 1968). Other workers have noted colds of a severe nature affecting a large proportion of men who had wintered, and who appeared to have little resistance to infection (Siple, 1960; Holmes, Allen, Bradburne & Stott, 1971). Totally different reactions in two groups of Antarctic wintering personnel exposed to the same cold within a few days of each other are recounted by Goldsmith in a personal communication to Wilson (1965). One group at Halley Bay suffered from severe colds, while the other group at Shackleton Base was symptom-free.

To resolve the differences resulting from these observations, and to gain a clearer picture of the epidemiology of viral respiratory disease in Antarctica, based upon detailed and objective observations amongst men at a number of bases, a prospective study was started in 1968, lasting for three years. During this time, men in Antarctica filled in symptom cards whenever respiratory symptoms occurred, both while they were at the bases and during the 6 months after they left to go home.

Logistics

The bases of the British Antarctic Survey are situated on or near the Graham-land Peninsula of Antarctica (Fig. 1). The summer season lasts from late November to late February, and each base is isolated for seven to eleven months, the southern bases having longer periods of isolation than the northern bases, mainly because of their inaccessibility. During the summer seasons 1969–71 the bases were visited by relief ships R.R.S. *John Biscoe*, R.R.S. *Shackleton* and M.V. *Perla Dan*, sometimes by the Royal Naval Vessel H.M.S. *Endurance* and occasionally by foreign

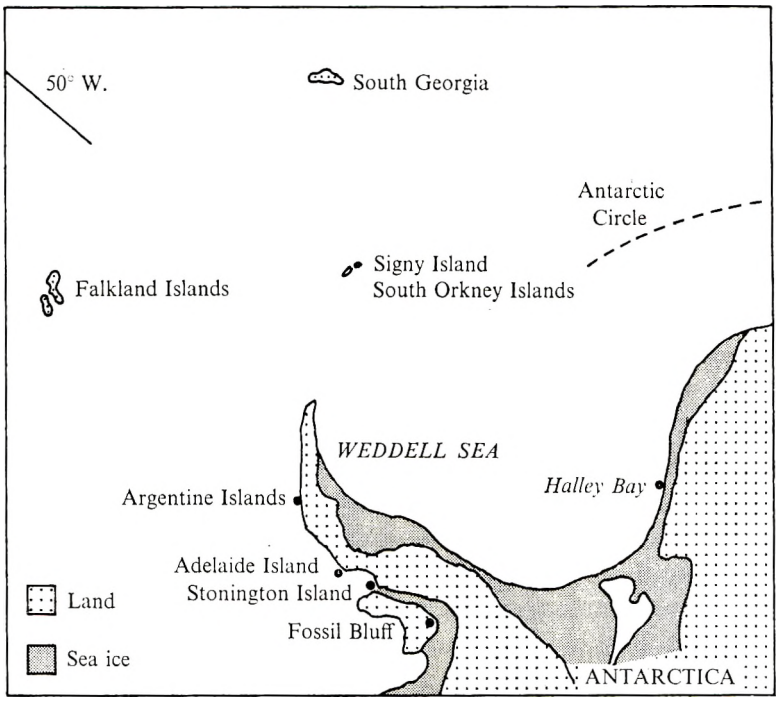


Fig. 1. The Antarctic Peninsula and adjoining sub-Antarctic regions.

ships. Three British bases, Adelaide Island, Stonington Island and Fossil Bluff bases were also served by two Survey aircraft from early December until March, and Halley Bay base was visited by aircraft from McMurdo Sound in addition to its relief ship, the M.V. *Perla Dan*.

METHODS

Symptom cards were completed daily by base members whenever respiratory symptoms appeared. They recorded blocked nose, running nose, sore throat, cough, tight chest, feeling tired, and headache, and the severity of each symptom was denoted by + for a mild symptom and + + for a severe symptom.

Colds were graded according to the types described by Tyrrell (1965). Abortive or doubtful mild colds were excluded.

For comparative purposes the severity of each cold was determined by adding up all the + signs recorded. A mean daily severity could then be derived.

Records obtained

Records were obtained from Halley Bay base of 30 men wintering in 1969, of 26 men wintering in 1970, and of 15 men of the 1969 wintering party during the 6 months after relief.

Records from Adelaide Island base include 12 men who wintered in 1969 and 6-month records of 7 of these who went home in early 1970.

Records from Stonington Island base and Fossil Bluff base, where interchange

of personnel occurred during the winter season, include 18 men who wintered in 1969 and 6-month records of five of these men who came home in early 1970.

Signy Island base members used the cards to produce continuous daily records of respiratory symptoms during 1968, 1969 and the relief periods after these years.

DESCRIPTION OF EPIDEMICS

Marguerite Bay

In early February 1969 the R.R.S. *John Biscoe* arrived in Marguerite Bay to replace stores and personnel at Adelaide Island, Stonington Island and Fossil Bluff bases. An epidemic of moderate to severe colds followed and has been described previously (Holmes *et al.* 1971).

Isolation started on 18 March 1969, and during the next 9 months no cold symptoms were observed in men at Stonington Island and Fossil Bluff bases. However, after 17 weeks of isolation at Adelaide Island base, moderately severe colds appeared in six out of 12 men 3 weeks after midwinter. Specimens taken during this outbreak have since been investigated in the laboratory, but no causative agent has been identified (Allen *et al.* 1973). No further colds occurred until after isolation was broken.

On 7 December 1969, two Survey aircraft arrived at Adelaide Island, introducing four extra men to the base, and two further men were brought into the area on 10 December. None of the incoming men had overt colds when they arrived at the base, nor did they develop colds while they were in Antarctica. One man had a running nose for 1 week after arrival, but this is not unusual in newcomers to Antarctica and was probably a response to unaccustomed cold air. He showed no other respiratory symptoms during his Antarctic stay.

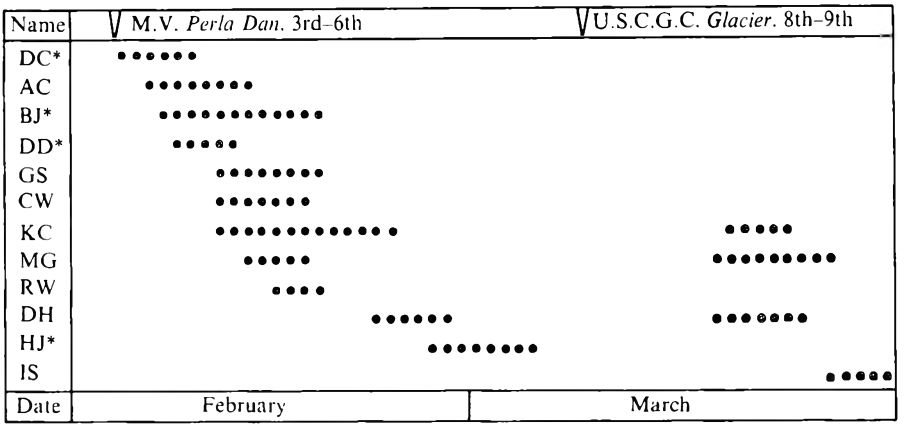
Two men who had wintered at Adelaide Island base developed symptoms of a moderately severe cold on 18 and 22 December 1969, but no other men at this base, or at the other Marguerite Bay bases were overtly affected, although interchange of personnel took place.

The R.R.S. *John Biscoe* arrived with stores and men in early February 1970, but this time, in contrast to the previous year, none of the men aboard were suffering from clinically apparent colds. The ship had taken 2 weeks to come down from the Argentine Islands because of bad sea-ice, but it is not known whether any men had colds at the start of this journey. No men who had wintered at the Marguerite Bay bases showed any common cold symptoms after the ship's arrival.

Symptoms in men who boarded the R.R.S. John Biscoe

Fourteen men, who had wintered in Antarctica, boarded the ship to go home. No colds occurred among these men until after the ship called at Argentine Islands base, where one man boarded her and showed symptoms of a moderately severe cold within 24 hr. Five men on the ship complained of similar symptoms in the next 3 days. The new man had been in contact with the Chilian ship *Yelcho* 2 weeks previously, but no records of colds at the base following the visit by this ship are available.

The *John Biscoe* later called at Signy Island base, 6 weeks after a call by the M.V. *Perla Dan*. An epidemic of colds had occurred among men at the base after



• = A day of symptoms in one man.
* = Newcomer to Antarctica.

Fig. 2. Respiratory symptoms occurring among 26 men at Halley Bay base in early 1970 in relation to visits by ships.

the M.V. *Perla Dan* left, but no men from the R.R.S. *John Biscoe* showed subsequent symptoms, though they worked with base members for 2 days. One assumes that the cold had died out, or that the conditions necessary for transmission and production of clinical disease were not present.

Among 14 men, in the 6 months following isolation, four recorded no colds, five recorded only one cold and five recorded two or three colds. In these last five men, the occurrence of the first cold did not appear to modify the severity of the second cold. No colds were observed during the 3-week voyage from Montevideo to Southampton, again demonstrating the effect of isolation on a small community.

Halley Bay

During the winter isolation of 1969 no colds occurred among 31 men. Two outbreaks of colds occurred during the relief period of early 1970 (Fig. 2).

The M.V. *Perla Dan* arrived at Halley Bay on 3 February 1970, thus breaking isolation, and stayed for 3 days. Men on the ship had suffered from recent colds (I. Leith, personal communication), and among the men put ashore was one man showing symptoms of a moderately severe cold, and three men who developed mild to moderately severe colds shortly after arrival.

During the next 3 weeks, 23 out of 31 men who had spent the previous winter at the base were sequentially affected with mild to moderately severe colds. Sixteen of these men suffered the colds after boarding the ship to go home, while seven of the men, who were staying a second year, suffered the colds while at base. There was no difference in the symptoms in the two groups. Symptoms in newcomers and men who had wintered in Antarctica were also essentially similar, but 15 out of the 23 men who had wintered and now had colds complained of sore throat, while this symptom was not present among newcomers with colds.

The second outbreak of common colds occurred 9 days after a visit by an American ship, the U.S.C.G.C. *Glacier* on 8 and 9 March, when there was inter-

change of personnel between the base and the ship. These were moderately severe colds, and there was little apparent difference in the symptom-complex between this outbreak and the previous one, but it is interesting to note that of the four men affected, all were men who had already wintered, and three of them had suffered colds in the previous epidemic. This previous experience appears to have had little protective effect in these cases.

After this second outbreak of colds 26 men were totally isolated from the outside world for the rest of 1970. Upper respiratory symptoms during this time were minimal, except in two men, in their second year in Antarctica, who showed symptoms at midwinter. One man complained of a blocked and running nose for 4 days, which on the first day was severe and accompanied by a sore throat. These symptoms started after playing midwinter football outside at low temperatures, and were probably secondary to irritation of the respiratory passages by the rapid inhalation of cold air. The second man complained of running nose for 3 days, severe at first, but becoming mild, associated with bad headache on the second and third day of the illness. These symptoms appeared 3 days after opening midwinter presents, which had been stored unopened since the last ship called. Other than these two men, no base members complained of symptoms resembling a common cold during the isolation period.

The occurrence of respiratory symptoms in these two men is the second example in this study of possible colds occurring during a long period of isolation. The appearance of symptoms in one man 3 days after opening midwinter presents, bears resemblance to reports of colds occurring among early Antarctic explorers after opening frozen blankets, and to the occurrence of colds in men at isolated camps, where virulent organisms in fur clothing are alleged to have been responsible for an outbreak of upper respiratory infection (Hedblom, 1961).

Colds among men leaving Halley Bay

Twenty-one men boarded the M.V. *Perla Dan* between 3 and 6 February 1970, all having spent at least one winter in Antarctica. Mild to moderately severe colds occurred in 16 of these men over a 2-week period starting 24 hr. after arrival of the ship at Halley Bay. Men were affected sequentially.

Colds were still occurring when the ship called at Signy Island base on 13 February 1970. No men at this base were suffering from colds when the ship called, but an outbreak of heavy colds occurred at the base within 2 weeks of this visit, without further ships calling.

On 19 February 1970, the ship arrived at Portstanley, Falkland Islands, and within 48 hr two men had developed colds, their second since relief, one mild and one moderately severe. During the 3-week voyage to Southampton no colds were recorded, reflecting the isolation of the men during the journey. Colds started to appear among the men 3 days after arrival at Southampton, and occurred throughout the summer months. Meanwhile, in Antarctica, colds had died out, leaving the majority of the men symptom-free for the rest of the isolation period (Fig. 3).

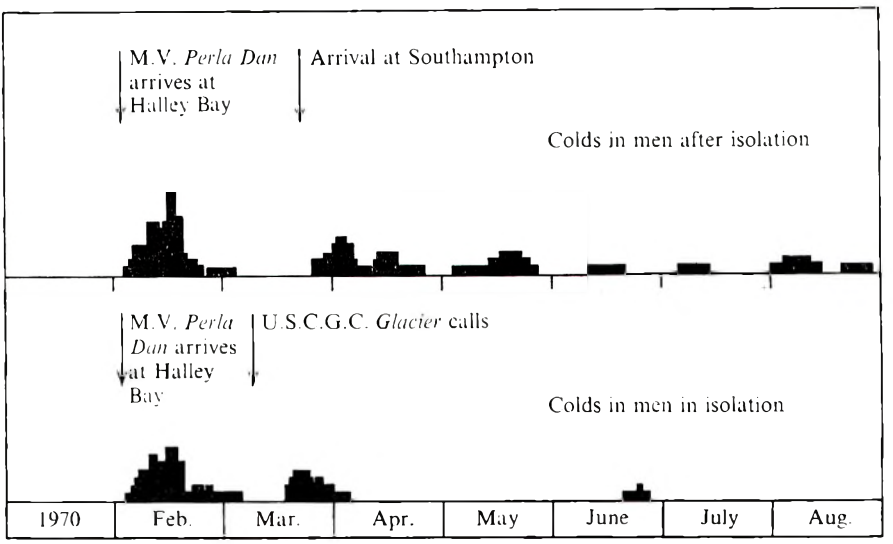


Fig. 3. Colds occurring among 21 men upon leaving the Antarctic and in 26 men who spent the winter in isolation in Antarctica. After isolation colds continued to appear among the men, while in Antarctica colds died out after the last ship had called.

Signy Island

In the two 7-month periods of isolation in 1968 and 1969 no colds occurred in 14 and 13 men respectively.

During the 1968/69 summer period of 5 months, when vessels called frequently, daily records showed only two colds in 23 men, both occurring in newcomers.

The records for the 1969/70 summer relief period are incomplete, but the base diary records the occurrence of heavy colds among the majority of the base personnel in the 3 weeks following the visit of the M.V. *Perla Dan*, on its way back from Halley Bay. As described already, men on the ship were at this time suffering from common colds.

ANALYSIS OF RECORDS

The study covered 110 man-years in Antarctica, during which time there were 69 colds recorded, an overall rate of 0·6 colds/man year.

During isolation periods, 112 men between them spent 898 months in groups of 4 to 31 men, not including the first month of each isolation period to allow for colds occurring after the visit of the last vessel. Eight colds were recorded, giving a rate of 0·1 colds/man year in isolation in Antarctica.

During the summer relief periods 140 men between them spent 348 months in Antarctica and recorded 61 colds a rate of 2·1 colds/man year.

Insufficient numbers were available for valid comparison of symptoms in newcomers and men who had wintered but generally the duration and severity of colds in the two groups were similar and there was no evidence of newcomers being particularly susceptible to colds.

Table 1. *Clinical features of colds in men who had wintered in Antarctica*

	Colds in Antarctica	Colds after contact with less-isolated communities
Total no. colds	45	34
Percentage of total with:		
Nose blocked	91	88
Nose running	87	94
Throat sore	78	86
Cough	38	65 ($P = < 0{\cdot}02$)
Tight chest	18	41 ($P = < 0{\cdot}05$)
Feeling tired	73	79
Headache	58	68
Median duration (days)	7·5	10·4 ($P = < 0{\cdot}05$)
Median severity of colds	21·4	35·3 ($P = < 0{\cdot}05$)
Median severity/day	2·9	3·6 ($P = > 0{\cdot}05$)

Symptoms in the two groups were compared using Yates's modification of the Chi-squared test, and duration and severity in the two groups were compared by analysis of variance (Holman, 1962).

The severity of colds suffered after return to less-isolated communities was significantly greater than that suffered in Antarctica when considered in terms of the total number of plus signs recorded during a cold, but comparison of colds in relation to the average number of pluses recorded each day showed no significant difference in severity.

In men who had wintered in Antarctica colds suffered after contact with less-isolated communities were generally similar in daily severity to those suffered while the men were still in Antarctica, but showed a greater incidence of cough and tight chest, and a longer duration (Table 1).

DISCUSSION

This study of acute respiratory infections among small groups of men during and after isolation in Antarctica has provided fairly clear evidence that in such an environment the common cold usually behaves as an infectious disease introduced from the outside. Occasionally, however, outbreaks of cold occur after many weeks of isolation, and presumably in such cases the responsible agents are preserved in some form in the environment, or in the respiratory tract cells of the isolated men.

The occurrence of colds on breaking isolation has been very variable. Colds of a moderate to severe nature have demonstrated the epidemic pattern most clearly, with colds starting soon after initial contact with newcomers and affecting a high proportion of men exposed. The epidemic pattern of mild colds has not been so clearly visible, with a long interval between initial contact and onset of the first cold, and only a few men affected. This could either mean that such mild colds do not transmit well under Antarctic conditions, or that men become infected without showing symptoms.

The high proportion of men involved in epidemics of moderately severe colds after wintering in Antarctica suggests that isolation predisposes to susceptibility

The longer duration of colds occurring after contact with less-isolated communities and the increased incidence of cough and tight chest may indicate a greater rate of secondary infection.

The overall rate for the occurrence of colds in men in Antarctica of less than one cold per man per year is similar to that seen in other isolated communities such as Tristan da Cunha (Shibli *et al.* 1971). The rate during long periods of isolation falls to 0·1 colds per man per year, and during the summer relief periods when communications are established, the rate of 2·1 colds per man per year approaches that seen in the less-isolated rural and urban communities from which the Antarctic personnel originate.

The author is indebted to members of the British Antarctic Survey for taking part in this study, to the medical officers who helped with the distribution and collection of cards, to Dr O. Edholm and Dr D. A. J. Tyrrell for advice and encouragement, and for providing space at the Clinical Research Centre, Harrow, and to the British Antarctic Survey for allowing access to records and for making the study possible.

REFERENCES

ALLEN, T. R., BRADBURNE, A. F., STOTT, E. J., GOODWIN, C. S. & TYRRELL, D. A. J. (1973). An outbreak of common cold at an Antarctic Base after seventeen weeks of complete isolation. *Journal of Hygiene* **71**, 657.

CAMERON, A. S. & MOORE, B. W. (1968). The epidemiology of respiratory infection in an isolated Antarctic community. *Journal of Hygiene* **66**, 427.

HEDBLOM, E. E. (1961). The medical problems encountered in Antarctica. *Military Medicine* **126**, 818.

HOLMAN, H. H. (1962). In *Biological Research Method*. London: Oliver and Boyd.

HOLMES, M. J., ALLEN, T. R., BRADBURNE, A. F. & STOTT, E. J. (1971). Studies of respiratory viruses in personnel at an Antarctic base. *Journal of Hygiene* **69**, 187.

SHIBLI, M., GOOCH, S., LEWIS, H. E. & TYRRELL, D. A. J. (1971). Common colds on Tristan da Cunha. *Journal of Hygiene* **69**, 255.

SIPLE, P. A. (1960). Living on the South Polar ice cap. In *Cold Injury*. Trans. Sixth Conf., Fort Knox (ed. S. M. Horvath), p. 89. New York: Josiah Macy, Jr. Foundation.

TAYLOR, I. M. (1960). Medical experiences at McMurdo Sound. In *Cold Injury*. Trans. Sixth Conf., 1958, Fort Knox (ed. S. M. Horvath), p. 117. New York: Josiah Macy, Jr. Foundation.

TYRRELL, D. A. J. (1965). In *Common Colds and Related Diseases*. London: Edward Arnold.

WILSON, O. (1965). Human adaptation to life in Antarctica. *Monographiae Biologicae*, no. 15, 690.

J. Hyg., Camb. (1973), **71**, 657
Printed in Great Britain

An outbreak of common colds at an Antarctic base after seventeen weeks of complete isolation

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(*Received* 28 *February* 1973)

SUMMARY

Six of 12 men wintering at an isolated Antarctic base sequentially developed symptoms and signs of a common cold after 17 weeks of complete isolation. Examination of specimens taken from the men in relation to the outbreak has not revealed a causative agent.

INTRODUCTION

It has commonly been believed that on small Antarctic bases, isolated for many months, upper respiratory infections die out during the first few weeks of isolation and that the men are virtually symptom-free for the rest of the isolation period. With the arrival of the relief ship cr aircraft, outbreaks of respiratory disease have been noted to occur (Taylor, 1960; Siple, 1960; Hedblom, 1961; Cameron & Moore, 1968; Holmes, Allen, Bradburne & Stott, 1971). This has tended to follow the pattern seen in other isolated communities (Paul & Freese, 1933; Shibli, Gooch, Lewis & Tyrrell, 1971).

Several studies of upper respiratory disease in men at isolated Antarctic stations have been undertaken. Sera obtained from the McMurdo Sound wintering party of 1958 were tested for the presence of antibodies to a number of respiratory viruses, but not including rhinoviruses, and showed no evidence of infection with any of the viral antigens tested (Chanock, R. M., quoted by Cameron & Moore, 1968). A systematic study of monthly serum specimens collected from the members of the South African National Antarctic Expeditions in 1961–62 showed no evidence of new virus infection (J. H. S. Gear, quoted by Cameron & Moore, 1968). In their 1968 study of the epidemiology of respiratory infections at Mawson, an Australian Antarctic Research Expedition station, Cameron & Moore (1968) made observations on infective diseases during the period of isolation, and found no diagnostic rises in antibody titre against influenza viruses A and B, mumps, adenovirus, herpes simplex and ornithosis. All attempts at virus isolation from throat, nose and faeces swabs were unsuccessful.

The apparent absence of respiratory infections for long periods during isolation in Antarctica has provided opportunity for basic epidemiological study, and

experimental inoculation of volunteers using easily traced viruses was started in 1968 with interesting results (Holmes *et al.* 1971). Further study along these lines was contemplated for the winter of 1969 at Adelaide Island Base, one of the British Antarctic Survey stations. During the preliminary observation period, after 17 weeks of isolation, upper respiratory symptoms occurred in one man and then spread to half the Base complement over the next week in the manner of an infectious disease. No virus had been artificially introduced at this time, and the outbreak was unexpected. Clinical records were kept, samples of serum and nasal washings were taken for analysis in the United Kingdom, and possible environmental factors were noted.

MATERIALS AND METHODS

Environment

The British Antarctic Survey Base on Adelaide Island (Latitude 67° S, Longitude 68° W) is situated on the south-west coast of the Antarctic Peninsula, and is about 1000 miles south of the Falkland Islands. The base acts as a centre for aircraft activities during the summer months, supporting remote bases and field parties working further south. Teams of husky dogs are sent to join the field parties, and set out soon after midwinter, with one man driving each team. Meteorologists make round-the-clock observations throughout the year.

The base is isolated from late March to mid-December each year, which coincides with the departure and arrival of the two Survey aircraft. There are one or two visits each summer from a relief ship.

The living quarters are dry wooden huts in the form of a small complex of buildings, heated by solid fuel burners, but the sleeping hut, made of fibreglass, tends to be damp and is heated with small electric convector heaters taking power from a diesel generator, and supplemented by paraffin stoves in the colder months. The sleeping hut is divided into cubicles and during the isolation period two men occupy each cubicle, with bunks 4 feet apart. The living rooms are more spacious, but because of the recurrent necessity for staying indoors because of inclement weather conditions, the men live in very close contact with each other.

Food comes mainly from packets or tins, and there are no fresh vegetables or fruit, but vitamin tablets are taken regularly. There is a daily bread bake, and special occasions merit the thawing of a small amount of meat from a $-20°$ C. freezer or from an ice cave in the nearby glacier. The water supply is from snow or ice blocks melted in stainless steel tanks. Waste from the sink drains under the living huts where it freezes, and is washed out with hoses during the summer melt. Ashes and sweepings are deposited in the sea in summer, and in the crack between sea-ice and land-ice during the winter, as are the contents of the chemical closets.

Weather conditions are variable, and changes often very rapid. Outside temperatures vary from a maximum just above zero centigrade, to a minimum of $-35°$ C. These temperatures relate to still air, and their cooling effect is much enhanced by increasing wind speeds. The average wind speed around the base area is about 14 knots. Frequent gales occur, often reaching wind speeds of 100 knots. Relative humidity outside during the year ranges from about 60 to 90 %.

At this latitude there is continual daylight during the summer months, and in winter months very little daylight or none at all. There are few animals present during the isolation period other than the husky dogs. The local Adelie penguins and most of the other bird life migrate north at the end of the summer season, leaving a small number of petrels and the occasional seal.

Logistics

In 1969, 14 men wintered at Adelaide Island Base. They arrived by sea and air at various times between December 1968 and March 1969, and relieved the previous complement of men. The Base members were all between the ages of 21 and 35 years, and were in good health. One of the men (I. W.) had spent the preceding winter at Adelaide Island Base, and two men had wintered on other Antarctic bases and transferred to Adelaide Island Base (M. B., B. T.).

The last aircraft left on 18 March and flew north for the winter, after which the Base was completely isolated from the outside world, except by radio.

Between 30 June and the time of the arrival of the first aircraft in early December, five husky dogs were present in the Base area. They were used for local transport and small expeditions into the field, and were fed on alternate days with seal meat from a pile of dead seals in the Base area.

Clinical data and specimen collection

The medical history of each Base member was noted throughout the period of isolation, and when symptoms and signs appeared they were recorded on daily observation charts as used at the Common Cold Research Unit at Salisbury, Wiltshire.

Serum and nasal samples were taken monthly from the men throughout the 8-month period of isolation, except during the outbreak of respiratory disease, when nasal secretions were taken on alternate days.

Serum from clotted blood specimens was stored in 2 ml. vials at -20 °C. in an electric freezer. Nasal secretions were collected by running 5 ml. sterile saline into each nostril and collecting the sample into sterilized disposable petri dishes. Samples were transferred from the petri dishes into duplicate vials containing 50 % nutrient broth and Ampicillin 0·1 mg., and stored at -20° C.

At the end of the isolation period, all samples were taken aboard the relief ship and stored for 10 weeks at 4° C., after which they were stored at -20 or -70 °C. at the Common Cold Research Unit at Salisbury.

Serological tests

Sera taken 3 weeks after the outbreak were examined at the Public Health Laboratory, Salisbury, for CF antibodies against common respiratory pathogens. Those sera showing high antibody titres were compared in further tests with sera taken 2 weeks before the outbreak.

Similar pairs of sera were examined using microtitre methods for CF antibodies against coronavirus OC43, 229E and MHV3, and for HI antibodies against

coronavirus OC43, coxsackievirus A21 and influenza viruses A2/Eng/12/64 and A2/Eng/344/68.

Sera for coronavirus CF and HI tests and coxsackievirus HI tests were inactivated at 56° C. for 30 min. Sera for influenza-virus HI tests were diluted 1/5 with cholera filtrate, left overnight at 37° C. and inactivated at 56° C. for 30 min. Sera taken 2 weeks before and 3 weeks after the outbreak were examined in the Microbiology Department, Northwick Park Hospital, for antistreptolysin-O antibodies and anti-DNase B antibodies.

Tissue and organ cultures

Virus isolation was attempted, from single samples and from pools of these samples, from the nasal secretions of those men who showed symptoms during the outbreak. A range of tissue cultures was used, grown in roller bottles at 37° C. and seeded onto glass tubes to produce a monolayer. Tubes were rolled at 33° C. after inoculation of 0·2 ml nasal secretion.

WI38 cells, MRC5 cells, L132 cells and human embryo kidney cells were maintained in Eagle's basal medium, containing 0·088% sodium bicarbonate, 2% fetal calf serum, and penicillin and streptomycin (100 i.u./ml.). Extra magnesium (0·03 M) was used in the maintenance medium for HeLa cells, and Eagle's medium containing 1% fetal calf serum, penicillin and streptomycin (100 i.u./ml.) and buffered with HEPES was used to maintain African green monkey kidney cells.

Organ cultures of human embryo nasal mucosa were prepared and maintained according to the method of Hoorn & Tyrrell (1969).

Broth cultures

Nasal washings from those men who showed symptoms were inoculated into Todd-Hewitt broth, and incubated at 37° C. for 7 days, to detect the presence of streptococci. The tubes were visually inspected every day, and were subcultured on the seventh day.

Volunteer experiments

These were carried out at the Common Cold Research Unit, Salisbury, Wiltshire, and the methods of study used there have been described previously (Andrewes, 1951). A pool of nasal secretions from men affected in the outbreak was inoculated into ten volunteers, and then a pool of nasal secretions from those volunteers was inoculated into a further seven volunteers. Sera were taken before and 2 weeks after inoculation.

RESULTS

Clinical observations

Between February and March 1969, new personnel arrived at Adelaide Island Base by air and ship from other parts of the Antarctic, and colds continued to occur in both old and new personnel.

The last aircraft left on 18 March and total isolation ensued. During the next 17 weeks no colds were observed by the 14 men at the Base who had been asked

to report any symptoms, and no respiratory disease was detected clinically. Two men left the Base with their dog teams at the end of June for 6 months, during which time they suffered from no respiratory symptoms.

Twelve men were left at the Base, and on 14 July 1969 one man presented with respiratory symptoms closely resembling those of a mild to moderately severe cold as described by Tyrrell (1965). During the next 2 weeks, eight out of the 12 men at the Base suffered similar respiratory symptoms, and a further two had attacks of sneezing. The symptoms and signs were charted (Fig. 1).

Quantitative assessment of nasal discharge by counting paper handkerchiefs used as suggested by Roden (1958) was largely impracticable, but was possible in one man and demonstrated a considerable increase in nasal discharge. In two men discharge was severe enough to warrant the use of two cotton handkerchiefs on the same day.

Of those affected, the average duration of symptoms was 5 days. The symptoms and signs of the outbreak did not resemble those of a streptococcal sore throat, as outlined by Christie (1969). Two men had unilateral posterior cervical adenitis of a mild nature.

Of the four men who showed minimal or no symptoms, two (H. B. and D. H.) worked in a separate hut during the daytime and shared the same sleeping cubicle, and one (J. N.) tended to work at night and sleep during the day, thus reducing contact with other Base members.

The man who first showed symptoms (M. B.) had spent the previous year at a four-man station at Fossil Bluff, further south, during which time no respiratory symptoms were noticed, but he had developed a cold in the few days after being transferred by air to Adelaide Island Base during the relief period.

The symptoms in one man (D. S.) may have been related to over-exertion in the cold air. He returned from a field trip 3 days after the start of the outbreak, and 2 days later complained of a stuffy nose and slightly increased nasal discharge lasting for 3 days. One day after this he developed a headache and a wheezy chest, with inspiratory and expiratory rhonchi in both lung fields. He recovered from the chest trouble in 1 week without the use of antibiotics and had no further chest complaints during the remainder of his stay. He gave no previous history of asthma or chest trouble.

Environmental observations

The outbreak occurred in the second half of July 1969, just over 3 weeks after midwinter, and was preceded by 2 weeks of bad weather conditions. In the first half of the month there were 5 days of gales, 12 days of blowing and drifting snow, and 7 days of snowfall. During this time most people stayed in the safety of the central hut, and social contact was thus greatest in the 2 weeks before the outbreak of respiratory disease.

There was minimal daylight for the first half of the month and there was no sunshine until 24 July, 10 days after the appearance of the first symptoms. With an average daily temperature of $-13{\cdot}4°$ C. the first half of the month was a little warmer than the last half, which gave an average daily temperature of $-17{\cdot}5°$ C.

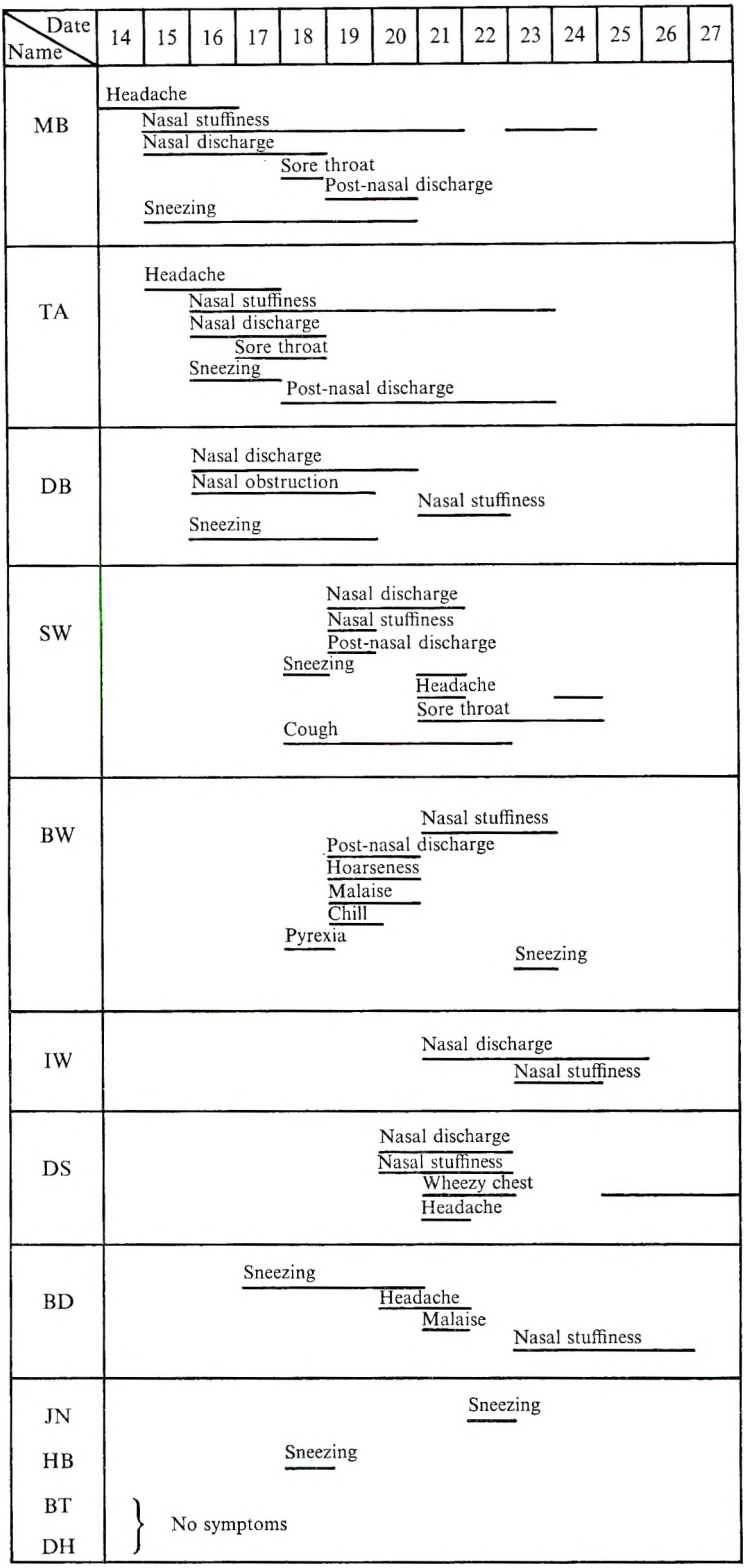

Fig. 1. Symptoms in 12 men in June 1969 after 17 weeks of isolation. The line under each symptom denotes the length of time that symptom was present.

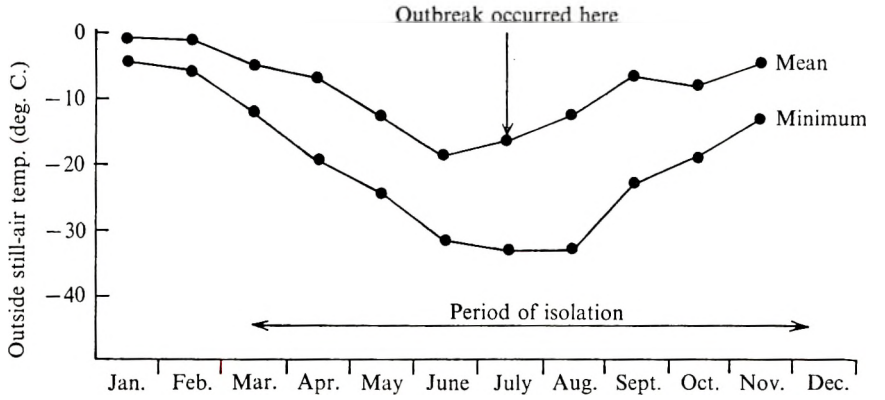

Fig. 2. Occurrence of outbreak in relation to mean and minimum monthly temperatures during 1969.

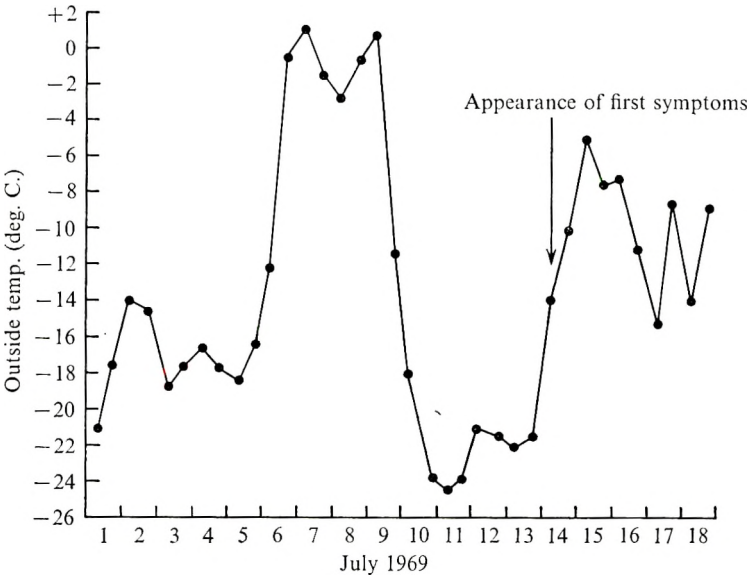

Fig. 3. Occurrence of outbreak in relation to bidaily temperatures during July 1969.

The timing of the outbreak in relation to the mean and minimum monthly temperatures is shown in Fig. 2, and in relation to outside temperatures taken at 6 a.m. and 6 p.m. during the first 18 days of July is shown in Fig. 3. A sudden change in weather between 9 and 10 July brought the outside temperature down from a high of 1·2° C. to a low of −24° C., and reduced the relative humidity outside from 93% to 79% over the same period.

Laboratory observations

Organ culture. In two experiments, in which pools of nasal washings were inoculated into cultures of human embryo nasal mucosa, there was no loss of ciliary activity compared with controls.

Tissue culture. Nasal washings from two men, who had been affected in the outbreak, produced cytopathic effects in WI38 cells, and these effects were reproduced in the next passage into WI38 cells. Although the effects resembled those produced by some coronaviruses, they could not be positively distinguished from non-specific cytopathic effects. Further passages into WI38, L132 and African green monkey kidney tissue cultures produced no cytopathic effects.

Harvests from the organ cultures produced no cytopathic effects in human embryo kidney cells, but passage from these into WI38 cells produced cytopathic effects again resembling those seen previously, which were not present in passed controls. Further passages did not produce any convincing cytopathic effects.

Electron microscope studies

Tissue culture cells from those tubes which showed cytopathic effects were examined with the electron microscope. Nc virus was seen.

Volunteer experiments

One doubtful cold was produced in one out of ten volunteers given pooled nasal secretions from the outbreak. Inoculation of a pool of nasal secretions from these volunteers into a further seven volunteers produced no symptoms.

Sera taken before and after inoculation from all volunteers were tested for coronavirus OC43 HI antibody, in view of the cytopathic effects seen in tissue culture. No rises in titre were demonstrated.

Broth cultures

Nasal washings taken from men with symptoms during the outbreak yielded no pathogenic bacteria.

Survey of CF antibodies against a range of respiratory pathogens

Sera from all the men at the Base taken 3 weeks after the start of the outbreak showed low or undetectable CF antibody titres against influenza B virus, respiratory syncytial virus, adenovirus, *Mycoplasma pneumoniae*, psittacosis, Q fever and coxsackie virus A.

Most of the sera had CF antibody titres of 1/20 against influenze A virus, but comparison with sera taken before the outbreak showed no rises in antibody titre. The sera of two men taken three weeks after the outbreak showed CF antibody titres of 1/100 and 1/50 against coxsackie virus B, but further comparison of similar pairs of sera showed no significant rises in CF antibody titre.

Further tests against specific viral and bacterial antigens

No HI antibody rises between paired sera, against the 1964 and 1968 strains of influenze virus A2, or against coronavirus OC43 were detected. The results of the CF antibody tests for coronavirus were obscured by anti-complementary activity and irreproducibility, and were thus unreliable. Further CF tests using sera treated with complement, and also sera treated with 1 % chloroform gave unsatisfactory results. No significant rises in anti-streptolysin-O antibodies were detected. AntiDNase B titres were all low or absent.

DISCUSSION

There is little doubt that an outbreak of respiratory disease occurred at an Antarctic base after 17 weeks of complete isolation. The symptoms occurring in six of 12 men were totally unexpected, and are most likely to have been of infective or allergic origin. The close resemblance of the symptoms to those of a common cold, the absence of common plant allergens, and the low level of dust in the huts, make it unlikely that the symptoms were of allergic origin. Finally, the sequential nature of the outbreak, suggesting person to person spread, indicates the presence of an infective agent.

A virus is most likely to have been the cause of this outbreak. However, in about 10 % of minor respiratory disease a β-haemolytic streptococcus may be discovered (Tyrrell, 1965). Our clinical and laboratory results indicate that streptococci were not involved. The range of viral agents responsible for the common cold syndrome is large, and the symptoms in each syndrome tend to overlap, and thus there are no sharp distinctions. Also the colds produced in Antarctica are not necessarily similar to those produced by the same viral agent under non-isolated conditions, so that the identification of a causative agent, on purely clinical grounds, is impossible. Viral antibody studies were limited to the range of respiratory antigens available, and HI and CF tests showed no evidence of infection with any of the viruses used. However, no studies of antibody against rhinovirus could be attempted, because of the multiplicity of serotypes, yet these viruses are thought to produce up to 50–60 % of upper respiratory infections in adults (Rhodes & van Rooyen, 1968), and could have been responsible for this outbreak.

Attempts at isolation of a viral agent using tissue and organ culture techniques were unfruitful, as was electron microscopy. The cytopathic effects seen in several passages of nasal washings in WI38 cells, although they appeared non-specific in character, could equally well have been the effects of a coronavirus, but such a possibility was not confirmed by serological tests on specimens taken during the outbreak and volunteer trials. Inoculation of volunteers with nasal washings taken during the outbreak produced negative results, and might indicate that if virus were present it was no longer viable, reflecting previous experience with such specimens (Cameron & Moore, 1968).

The occurrence of a common cold during isolation, when the chances of introduction of new infection from the outside are virtually nil, implies that in some way virus persisted, either in the environment or in the men. The possibility that husky dogs act as an animal reservoir of human respiratory virus was suggested by Holmes *et al.* (1971), but subsequent studies in Antarctica have failed to support this hypothesis (Allen & Holmes, in preparation). In addition, no common human respiratory virus is known to have been transmitted from an animal host to man with the production of disease and it seems unlikely that animals were involved in causing this outbreak.

Persistence of respiratory virus on inanimate objects must be considered. At midwinter many new boxes were opened, and suits were worn which contained

soiled but usable handkerchiefs. Colds have occurred at this time among other men isolated in Antarctica (Allen, in preparation), but in contrast to the present outbreak the colds did not apparently spread. The interval of 3 weeks between midwinter and the start of the outbreak makes it unlikely that infection occurred at midwinter, especially as the two men who left the Base soon after the celebrations had finished showed no evidence of respiratory disease.

Virus might have persisted in the respiratory tract of one or more men at the Base. If such were the case it would be necessary to postulate a triggering mechanism to precipitate symptoms, and it is interesting to note that symptoms occurred 4 days after a precipitous fall in outside temperature, and during one of the coldest months of the year, which Hope-Simpson (1958) and Lidwell, Morgan & Williams (1965) have correlated with increased incidence of respiratory disease. There is disagreement about whether the viruses which cause common colds can be carried by adults, and how important this is in epidemiology. The pattern of virus infection revealed by long-term studies, such as the virus watch programme (Elveback *et al.* 1966), is of a series of short infections with different viruses, and in the case of influenza virus disappearance of the current strain when a new serotype appears. On the other hand, adenoviruses may be shed by children for periods of months and recovered from the tonsils in a high proportion of cases, without evidence of acute respiratory infection, and non-respiratory viruses such as those of the herpes group often persist for the lifetime of a man. Furthermore, observations in animals have shown that pigs can carry swine influenza and transmit infection to other pigs 3 months later (Blaškovič *et al.* 1970), turkeys may carry and shed influenza virus A after apparent recovery (Robinson, Easterday & Tumova, 1972), and cattle which have recovered from foot and mouth disease still reproduce virus in the pharynx, and can initiate epidemics on contact with non-immune cattle (Graves *et al.* 1971).

There are thus precedents in both children and animals for persistence of respiratory viruses, but in adults the laboratory evidence for carriage and reactivation of common cold viruses is weak. It may be that such evidence can only be found in rather unusual conditions of isolation and stress, such as occur in Antarctica. It is likely to be a rare phenomenon, but it might well be important in explaining the persistence of the large number of rhinovirus serotypes which make an appearance in many areas when the temperature falls.

We wish to thank members of the British Antarctic Survey, and volunteers and staff at the Common Cold Research Unit, Salisbury, for helping with this study. We also wish to thank Mrs R. Pasmore, Mrs P. K. Brown and Miss B. Somerset for technical help, Dr R. R. Dourmashkin for E.M. studies, and the British Antarctic Survey for making the study possible.

REFERENCES

ANDREWES, C. H. (1951). The Common Cold. *Scientific American* **184**, no. 2, 39.

BLAŠKOVIČ, D., JAMRICHOVÁ, O., RATHOVÁ, V., KOČIŠKOVÁ, D. & KAPLAN, M. M. (1970). Experimental infection of weanling pigs with A/swine influenza virus. 2. The shedding of virus by infected animals. *Bulletin of the World Health Organization* **42**, 767.

CAMERON, A. S. & MOORE, B. W. (1968). The epidemiology of respiratory infection in an isolated Antarctic community. *Journal of Hygiene* **66**, 427.

CHRISTIE, A. B. (1969). *Infectious Diseases: Epidemiology and Clinical Practice*, p. 290. E. & S. Livingstone Ltd: Edinburgh and London.

ELVEBACK, L. R., FOX, J. P., KETLER, A., BRANDT, C. D., WASSERMAN, F. E. & HALL, C. E. (1966). The virus watch programme: A continuing surveillance of viral infections in metropolitan New York families. III. Preliminary report on association of infections with disease. *American Journal of Epidemiology* **83**, 436.

GRAVES, J. H., MCVICAR, J. W., SUTMOLLER, P., TRAUTMAN, R. & WAGNER, G. G. (1971). Latent viral infection in transmission of foot and mouth disease by contact between infected and susceptible cattle. *Journal of Infectious Diseases* **124**, 270.

HEDBLOM, E. E. (1961). The medical problems encountered in Antarctica. *Military Medicine* **126**, 818.

HOLMES, M. J., ALLEN, T. R., BRADBURNE, A. F. & STOTT, E. J. (1971). Studies of respiratory viruses in personnel at an Antarctic base. *Journal of Hygiene* **69**, 187.

HOORN, B. & TYRRELL, D. A. J. (1969). Organ cultures in virology. *Progress in Medical Virology* **11**, 408. Karger: Basel and New York.

HOPE-SIMPSON, R. E. (1958). Discussion on the common cold. *Proceedings of the Royal Society of Medicine* **51**, 267.

LIDWELL, O. M., MORGAN, R. W. & WILLIAMS, R. E. O. (1965). The epidemiology of the common cold. IV. The effect of weather. *Journal of Hygiene* **63**, 427.

PAUL, J. H. & FREESE, H. L. (1933). An epidemiological and bacteriological study of the 'common cold' in an isolated Arctic community (Spitzbergen). *American Journal of Hygiene* **17**, 517.

RHODES, A. J. & VAN ROOYEN, C. E. (1968). *Textbook of Virology*. Fifth edition, p. 262. The Williams and Wilkins Co.: Baltimore.

ROBINSON, G. H., EASTERDAY, D. C. & TUMOVA, B. (1972). Influenza and other respiratory viruses of man and animals. *Proceedings of the 2nd International Congress for Virology*, Budapest 1971, p. 120, ed. J. L. Melnick. Karger: Basel.

RODEN, A. T. (1958). Discussion on the common cold. *Proceedings of the Royal Society of Medicine* **51**, 271.

SHIBLI, M., GOOCH, S., LEWIS, H. E. & TYRRELL, D. A. J. (1971). Common colds on Tristan da Cunha. *Journal of Hygiene* **69**, 255.

SIPLE, P. A. (1960). Living on the South Polar ice cap. In *Cold Injury*. Trans. Sixth Conf., 1958, Fort Knox (ed. S. M. Horvath), p. 89. New York: Josiah Macy, Jr. Foundation.

TAYLOR, I. M. (1960). Medical experiences at McMurdo Sound. In *Cold Injury*. Trans. Sixth Conf., 1958, Fort Knox (ed. S. M. Horvath), p. 139. New York: Josiah Macy, Jr. Foundation.

TYRRELL, D. A. J. (1965). *Common Colds and Related Diseases*. London: Edward Arnold.

J. Hyg., Camb. (1973), **71**, 669
Printed in Great Britain

Trimethoprim-resistance and its transferability in *E. coli* isolated from calves treated with trimethoprim-sulphadiazine: a two year study

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(*Received* 2 *March* 1973)

SUMMARY

Regular examination of rectal swabs revealed the presence of very low numbers of trimethoprim resistant *Escherichia coli* in the faeces of 10 batches of calves successively reared in the same shed and none of these strains transferred trimethoprim resistance to *E. coli* K12. All the calves had received oral doses of 30 mg/kg day of trimethoprim-sulphadiazine for 5 consecutive days. From two subsequent batches of calves reared in the same shed, however, several isolations were made of *E. coli* with transmissible R factors determining trimethoprim and streptomycin resistance. Shortly before these strains were detected, isolations of *E. coli* with similar properties had been made from other calves, in a different shed, which had been fed much higher doses of trimethoprim-sulphadiazine. Serological evidence indicated that all the *E. coli* isolated carrying this R factor belonged to the same strain, which had apparently spread from the second shed to the first. No evidence of 'in vivo' transfer of the R factor to other enteric bacteria was obtained.

INTRODUCTION

Trimethoprim was first made available for general use in human medicine in 1968 and released for use in veterinary medicine in 1969. It is a synthetic antibacterial drug which inhibits the enzyme dihydrofolate reductase, necessary for the essential synthesis, by bacteria, of folinates. Therapeutic preparations of trimethoprim are always combined with a sulphonamide because of the strong synergistic action exhibited by their combination (Bushby, 1969). The combination of trimethoprim and sulphonamide has proved useful in the treatment of a wide range of bacterial infections in man (Hughes, 1969; Seboulet, 1971; Kabbage, Ben Brahim & Amine, 1971) and in animals (Rehm & White, 1970; Rail & Kaller, 1971; Scholl *et al.* 1971).

One important aspect of the use of the combination in human medicine has been the low incidence of trimethoprim-resistant coliform bacilli encountered since its introduction 4 years ago (McAllister *et al.* 1971; Gillespie, Lee, Linton & Rowland, 1971; Fleming, Datta & Grüneberg, 1972; Lacey, Gillespie, Bruten & Lewis, 1972). Recently, however, a number of strains of *Klebsiella* spp. and

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E. coli, of human origin, were discovered harbouring transmissible R factors determining high levels of resistance to both trimethoprim and sulphonamide (Fleming *et al.* 1972; Datta & Hedges, 1972). Little information has been published on the incidence and transferability of trimethoprim-resistance in bacteria of animal origin. This report describes the incidence of trimethoprim-resistant strains of *E. coli* encountered in batches of calves treated with trimethoprim-sulphadiazine, together with the isolation and epidemiology of a strain of *E. coli* harbouring a transmissible R factor determining resistance to both trimethoprim and streptomycin.

MATERIALS AND METHODS

Source of samples

During the period between 1 October 1970 and 9 August 1972 twelve successive batches of 28 one-week-old Friesian calves were kept in the same building (Shed 1) in adjacent individual pens and fed milk replacer once daily. Each batch of calves was reared in Shed 1 for 6 weeks and then moved out into nearby covered yards for fattening and subsequent sale. After the removal of each batch of calves, the interior of Shed 1 was thoroughly cleaned, disinfected with caustic soda solution and left empty for 2 weeks. All calves in each batch received trimethoprim-sulphadiazine,* prophylactically, in their milk replacer for five consecutive days beginning 48 hr. after their arrival. The dose, 30 mg/kg day, was that normally recommended for the treatment of infection. Rectal swabs were taken from each calf upon arrival and at weekly intervals until removal.

During the period between 14 April 1972 and 16 May 1972 an additional batch of 18 one-week-old Guernsey calves (Batch A) were monitored. All calves in Batch A were housed in a building (Shed 2) which was separated from Shed 1 by a distance of approximately 100 yards. All calves were kept in adjacent individual pens, fed milk replacer for 5 weeks, and then sold. On arrival, and as part of an experimental study, Batch A was divided in three groups (Groups 1, 2 and 3). Groups 2 and 3 received high doses of trimethoprim-sulphadiazine† corresponding to 60 mg/kg day and 150 mg/kg day respectively for eight consecutive days. Group 1 did not receive any treatment and the animals were used as controls. Rectal swabs were taken from each calf in all groups, every 2 days, until treatment ended and from then on at weekly intervals.

Trimethoprim-resistant E. coli

Strains of trimethoprim-resistant *Escherichia coli* were isolated by streaking each rectal swab on a plate of 'Wellcotest' Sensitivity Testing Agar‡ (W.S.T.), containing 1 % (w/v) lactose, 0·005 % (w/v) neutral red and 1·0 μg. trimethoprim per ml. All plates were incubated at 37° C. for 24 hr. and examined for lactose-fermenting colonies. One lactose-fermenting colony, having typical morphological features, was picked off each plate and initially identified by streaking on MacConkey agar. Each culture was then subjected to further biochemical tests.

* (Tribrissen Dispersible Powder, Burroughs Wellcome & Co.)
† (Tribrissen Bolus – Burroughs Wellcome & Co.)
‡ (Wellcome Reagents Ltd., Beckenham, Kent.)

All cultures identified as *E. coli* were tested for their sensitivity, by means of Oxoid 'Multodisks' to ampicillin (A); streptomycin (S); chloramphenicol (C); tetracycline (T); neomycin (N); furazolidone (F); sulphonamide (Su); trimethoprim-sulphamethoxazole (1:20; 25 μg.) and with individual disks containing 1·25 μg. trimethoprim (TMP). The medium used was W.S.T. agar. Strains with no zone of inhibition around the trimethoprim disks were recorded as resistant. The minimum inhibiting concentrations (M.I.C.) of trimethoprim for these strains were then determined by streaking small inocula on plates of W.S.T. agar containing serial dilutions of the drug. The inocula were standardized by diluting broth cultures sufficiently to produce about 20 colonies per inoculum.

Demonstration of R factors in strains of trimethoprim-resistant Escherichia coli

Each strain of trimethoprim-resistant *E. coli* was grown in mixed broth culture with a nalidixic acid-resistant *E. coli* K 12 recipient. The mixtures were then plated on a selective medium of W.S.T. agar containing 1 % (w/v) lactose, 0·005 % (w/v) neutral red, nalidixic acid (100 μg./ml.), and either streptomycin (25 μg./ml.), chloramphenicol (25 μg./ml.), ampicillin (25 μg./ml.) or trimethoprim (5·0 or 1·0 μg./ml.). The recipient used was *E. coli* K 12, non-lactose fermenting and sensitive to antibacterial drugs other than nalidixic acid. Neither donor nor recipient grew on the selective medium. Colonies developing from the mixed cultures were streaked on MacConkey agar plates, identified as *E. coli* K 12 by their colonial morphology and inability to ferment lactose, and tested for their sensitivity to a range of antibacterial drugs including trimethoprim.

Serological tests

All cultures of *E. coli*, harbouring transmissible R factors determining trimethoprim-resistance, were serologically tested for antigenic similarity.

Four representative cultures were chosen and used to prepare OK antisera in rabbits (Sojka, 1965). All cultures, including the four immunizing cultures, were then tested for agglutination by each of the four antisera, using conventional tube methods with either live, boiled or autoclaved suspensions of each culture (Sojka, 1965). The titres of each set of agglutinations were recorded.

The four immunizing cultures were serotyped by Dr B. Rowe, Central Public Health Laboratory, Colindale.

Enterotoxin tests

The four immunizing cultures were tested for their ability to produce enterotoxin using the ligated gut test in calves (Smith & Halls, 1967). In addition, following transfer of the R factor determining trimethoprim-resistance, a culture of *E. coli* K 12 and of a known recipient of enterotoxin plasmids *E. coli* O8:K$^-$:H$^-$ (referred to as strain D 282 by Smith & Linggood, 1972) were tested using the same technique, to determine the possibility of a link between enterotoxin production and the R factor.

E. coli O8:K$^-$:H$^-$ with and without the R factor determining trimethoprim-resistance, was also tested by Mr W. J. Sojka, Central Veterinary Laboratory,

Table 1. *Trimethoprim-resistant* Escherichia coli *isolated from Batch* 10 *in Shed* 1 *between* 1 *March and* 12 *April* 1972

No. of weeks after arrival of calves	Total no. of calves from which cultures were isolated	Resistance patterns of cultures isolated	No. of cultures with specified resistance pattern	M.I.C. of TMP for each culture (μg/ml)	Drug resistance transferred to *E. coli* K12 from each culture
0	0	—	—	—	—
1*	8	S, C, T, A, TMP, Su	5	16·0	C, T, Su
		S, T, N, A, TMP, Su	1	3·0	None
		S, T, A, TMP, Su	1	12·0	None
		S, A, TMP, Su	1	4·0	None
		No further trimethoprim-resistant *E. coli* isolated			

* End of treatment period.

S, Streptomycin; C, chloramphenicol; T, tetracycline; N, neomycin; A, ampicillin; F, furazolidone; Su, sulphonamide; TMP, trimethoprim.

Weybridge, for antigen 'KCO', whose presence is correlated with enteropathogenicity in calves (Smith & Linggood, 1972).

RESULTS

Incidence and epidemiology of trimethoprim-resistant Escherichia coli *encountered in the calves*

No trimethoprim-resistant *E. coli* were isolated from nine successive batches of calves reared and monitored in Shed 1 during the first 17 months of the study. Trimethoprim-resistant *E. coli* were isolated, however, from the next three batches of calves (Batch Nos. 10, 11 and 12) reared in Shed 1 and details of these strains are summarized in Tables 1, 2 and 3 respectively. Tables 2 and 3 show that the majority of cultures isolated from Batches 11 and 12 transferred trimethoprim-resistance to *E. coli* K12. These were very prevalent in the faecal flora of the calves shortly after the end of treatment, and remained so even when samples were taken 3 or 4 weeks later. There were no serious outbreaks of scouring in any of the batches monitored in Shed 1.

No trimethoprim-resistant *E. coli* were isolated from the calves in Batch A until shortly after the end of treatment. Details of the trimethoprim-resistant *E. coli* isolated from calves in this batch, after treatment are summarized in Table 4. No trimethoprim-resistant *E. coli* were isolated from the untreated group of calves (Group 1). A serious outbreak of scouring occurred in all groups of calves in Batch A together with some deaths; bacteriological findings, however, did not implicate trimethoprim-resistant *E. coli* as the causative pathogen.

Strains of *E. coli* with transferable trimethoprim-resistance were first isolated from calves in Batch A which had received 150 mg. trimethoprim-sulphadizine/kg day (Group 3). These calves were reared in Shed 2 at approximately the same time as Batch 11 in Shed 1. It therefore seems likely that these strains were, in some way, transferred from Shed 2 to Shed 1 where they subsequently re-emerged

Table 2. *Trimethoprim-resistant* Escherichia coli *isolated from Batch* 11 *in Shed* 1 *between* 25 *April and* 6 *June* 1972

No. of weeks after arrival of calves	Total no. of calves from which cultures were isolated	Resistance patterns of cultures isolated	No. of cultures with specified resistance pattern	M.I.C. of TMP for each culture (μg/ml)	Drug resistance transferred to *E. coli* K12 from each culture
0	2	C, A, TMP, F	1	3·0	None
		S, C, T, A, TMP, Su	1	2·0	None
1*	3	S, C, A, TMP, Su	1	> 1000	All
		S, C, T, A, TMP, Su	1	> 1000	All
		S, TMP, Su	1	4·0	None
2	17	S, C, A, TMP, Su	7	> 1000	All
		S, C, T, A, TMP, Su	9	> 1000	All
		S, A, TMP, Su	1	3·0	None
3	9	S, C, A, TMP, Su	3	> 1000	All
		S, C, T, A, TMP, Su	6	> 1000	All
4	2	S, C, A, TMP, Su	1	> 1000	All
		S, C, T, A, TMP, Su	1	> 1000	All
5	4	S, C, A, TMP, Su	1	> 1000	All
		S, C, T, A, TMP, Su	3	> 1000	All
6	4	S, C, A, TMP, Su	2	> 1000	All
		S, C, T, A, TMP, Su	2	> 1000	All

* End of treatment period.

Table 3. *Trimethoprim-resistant* Escherichia coli *isolated from Batch* 12 *in Shed* 1 *between* 28 *June and* 9 *August* 1972

No. of weeks after arrival of calves	Total no. of calves from which cultures were isolated	Resistance patterns of cultures isolated	No. of cultures with specified resistance pattern	M.I.C. of TMP for each culture (μg/ml)	Drug resistance transferred to *E. coli* K12 from each culture
0	1	C, A, TMP, Su	1	15·0	None
1*	25	S, C, A, TMP, Su	24	> 1000	All
		S, C, T, A, TMP, Su	1	> 1000	All
2	17	S, C, A, TMP, Su	16	> 1000	All
		S, C, T, A, TMP, Su	1	> 1000	All
3	8	S, C, A, TMP, Su	8	> 1000	All
4	6	S, C, A, TMP, Su	5	> 1000	All
		S, C, T, A, TMP, Su	1	> 1000	All
5	3	S, C, A, TMP, Su	3	> 1000	All
6	2	S, C, A, TMP, Su	2	> 1000	All

* End of treatment period.

Table 4. *Trimethoprim-resistant* Escherichia coli *isolated from Batch A in Shed 2 between 14 April and 16 May 1972*

No. of weeks after end of treatment period	Total no. of calves from which cultures were isolated	Resistance patterns of cultures isolated	No. of cultures with specified resistance pattern	Dose rate of TMP/Su given to calves from cultures originated (mg/kg day)	M.I.C. of TMP for each culture (μg/ml)	Drug resistances transferred to *E. coli* K12 from each culture
0*	5	S, C, A, TMP, Su	1	150	> 1000	All
		S, C, A, T, TMP, Su	2	150	> 1000	All
		TMP, Su	1	60	4·0	None
		T, TMP, Su	1	150	4·0	None
1	5	S, C, A, TMP, Su	1	150	> 1000	All
		S, C, A, T, TMP, Su	2	150	> 1000	All
		S, A, TMP, Su	1	60	4·0	S, Su
		T, TMP, Su	1	150	4·0	None
2	2	S, C, A, TMP, Su	1	150	> 1000	All
		S, C, A, T, TMP, Su	1	150	> 1000	All
3	2	S, C, A, TMP, Su	1	150	> 1000	All
		S, C, A, T, TMP, Su	1	150	> 1000	All

* 24 hr after last dose of trimethoprim-sulphadiazine administered.

in the faeces of calves in Batch 11 after treatment. Disinfection of Shed 1, after the removal of Batch 11, did not prevent the re-emergence of these strains in the following batch of calves (Batch 12) after treatment.

Transfer of trimethoprim-resistance

The transferable drug resistances possessed by each strain of trimethoprim-resistant *E. coli* are shown in Tables 1–4. The only strains with transferable trimethoprim-resistance were those resistant to at least 1000 μg. trimethoprim per ml. These strains had either of two resistance patterns, S, C, T, A, Su, TMP or S, C, A, Su, TMP. When these cultures were used as donors of drug resistances and selection was made for either streptomycin, chloramphenicol or ampicillin resistance, every colony of converted recipient tested had all the donor's drug resistant markers *except TMP*. When trimethoprim resistance was selected for, however, all colonies of converted recipients tested had one of three resistance patterns: S, C, T, A, Su, TMP; S, C, A, Su, TMP or S, TMP. When recipient colonies received either S, C, T, A, Su, TMP or S, C, A, Su, TMP their M.I.C.'s of trimethoprim and streptomycin were always > 1000 μg./ml. and > 25 μg./ml. respectively. When recipient colonies received S, TMP, however, their M.I.C.'s of trimethoprim and streptomycin were always > 1000 μg./ml. and 10 μg./ml. respectively. These results suggest that each wild strain of *E. coli* had several different transmissible R factors, one of which determined resistance to both trimethoprim and streptomycin.

Frequencies of transfer of trimethoprim-resistance

The frequency of transfer of trimethoprim-resistance from the wild strain of *E. coli* to *E. coli* K 12 during overnight incubation of mixtures was approximately 5×10^{-8} per potential donor cell.

Serological tests on cultures of Escherichia coli *with transferable trimethoprim-resistance*

All live, boiled, and autoclaved suspensions of each culture tested, agglutinated to titres of 1/100, 1/100 and 1/400 respectively in all four OK antisera. All four immunizing cultures belonged to the same serotype; O101:K?(A):H37. It therefore seems likely that all the cultures tested belonged to the same serotype as the immunizing cultures.

Enterotoxin tests

All four immunizing cultures dilated segments of calf intestine and were therefore considered to be capable of enterotoxin production. Both *E. coli* K 12 and *E. coli* O8:K$^-$:H$^-$ each containing the R factor determining trimethoprim-streptomycin resistance did not dilate segments of calf intestine. It was therefore assumed that the R factor was not directly associated with enterotoxin production.

No extra antigenic component was detected in *E. coli* O8:K$^-$:H$^-$ on acquisition of the trimethoprim-streptomycin R factor (W. J. Sojka, personal communication).

DISCUSSION

The incidence of trimethoprim-resistant *E. coli* encountered during the first 18 months of the study was very low and in no case was trimethoprim resistance transmissible. During the remaining 5 months of the study, however, a large number of isolations of trimethoprim-resistant *E. coli* were made. The majority of these strains grew on a medium containing 1000 μg. trimethoprim/ml. All the strains which did so transferred similar degrees of trimethoprim resistance to *E. coli* K 12 in mixed culture. In each of the wild strains trimethoprim and sulphonamide resistance was determined separately by two different transmissible R factors. The R factor determining trimethoprim-resistance also determined streptomycin resistance. This R factor was designated a prototype of a new compatibility class, B (Hedges, Datta & Fleming, 1972); however, its designation has subsequently been changed to compatibility class Iβ, since it determines I pili (Hedges & Datta, 1973).

The hosts for the R factors determining trimethoprim-streptomycin resistance and sulphonamide resistance were all typical strains of *E. coli*. All had similar drug resistance patterns and had similar serological reactions. All were isolated from animals on one experimental station. It therefore seems likely that all were members of a single clone. This strain first emerged in a group of calves treated with large doses of trimethoprim-sulphadiazine. It seems likely that the strain was present in some of these calves' intestines before treatment, but in numbers

so low as to be rendered undetectable by the method of sampling. The subsequent proliferation of the strain in these calves was probably due to a strong selective pressure created by the high levels of drug administered. The strain subsequently spread to a different building where it became prevalent and persistent for several weeks in the faeces of a group of calves treated with normal doses of trimethoprim-sulphadiazine. This evidence, therefore, demonstrates how R factors determining trimethoprim and sulphonamide resistance in a host bacterium can lead to the rapid proliferation of that host when the combination is present in the environment. Such a situation would be expected to increase the chances of both R factors spreading to other strains of bacteria, for example *E. coli*. There was no evidence, however, to suggest that the latter had occurred in this study. This result may have been connected with the host strain's in vitro ability to transfer the R factor determining trimethoprim-streptomycin resistance to *E. coli* K12 at only very low frequencies.

It appears that R factor-determined trimethoprim-resistance in *E. coli* isolated from animals is, at present, a very rare phenomenon. The widespread use of trimethoprim in animals may encourage the spread of these R factors, but until now no such spread has become apparent in *E. coli*. I hope that other workers will continue to monitor the situation and report any changes which may occur in the prevalence of trimethoprim-resistance and its transferability in bacteria of animal origin.

I am grateful to Dr N. Datta, Dr B. Rowe, Mr G. White, Mr G. R. Craig, Dr L. Nagy and Dr H. Williams-Smith for help and assistance in various ways, and to Dr B. Rowe for serotyping strains of *E. coli*.

REFERENCES

BUSHBY, S. R. M. (1969). The combined antibacterial action in vitro of trimethoprim and sulphonamides. *Postgraduate Medical Journal* **45**, Suppl. 10.

DATTA, N. & HEDGES, R. W. (1972). Trimethoprim resistance conferred by W plasmids in Enterobacteriaceae. *Journal of General Microbiology* **72**, 349.

FLEMING, M. P., DATTA, N. & GRÜNEBERG, R. N. (1972). Trimethoprim resistance determined by R factors. *British Medical Journal* **1**, 726.

GILLESPIE, W. A., LEE, P. A., LINTON, K. B. & ROWLAND, A. J. (1971). Antibiotic resistance of coliform bacilli in urinary infections. *Lancet* ii, 675.

HEDGES, R. W. & DATTA, N. (1973). Plasmids determining I pili constitute a compatibility complex. *Journal of General Microbiology* **77**, 19.

HEDGES, R. W., DATTA, N. & FLEMING, M. P. (1972). R factors conferring resistance to trimethoprim but not sulphonamides. *Journal of General Microbiology* **73**, 573.

HUGHES, D. T. C. (1969). Single-blind comparative trial of trimethoprim-sulphamethoxazole and ampicillin in the treatment of exacerbations of chronic bronchitis. *British Medical Journal* **4**, 470.

KABBAGE, D., BEN BRAHIM, M. & AMINE, M. (1971). Trial of trimethoprim-sulphamethoxazole in the pathology of infections and in particular in purulent meningitis, typhoid and paratyphoid. *Journal Medical Marocain* **7**, 11.

LACEY, R. W., GILLESPIE, W. A., BRUTEN, D. M. & LEWIS, E. L. (1972). Trimethoprim-resistant coliforms. *Lancet* i, 409.

MCALLISTER, T. A., PERCIVAL, A., ALEXANDER, J. G., BOYCE, J. M. H., DULAKE, C. & WORMALD, P. J. (1971). Multicentric study of sensitivities of urinary tract pathogens. *Postgraduate Medical Journal* **47**, Suppl. 7.

Rail, W. & Kaller, H. (1971). A contribution from practice on the effectiveness of the trimethoprim/sulphonamide principle in Bavarian cattle holdings with bacterial problems in rearing. *Praktische Tierarzt* **52**, 10

Rehm, W. F. & White, G. (1970). A field trial with trimethoprim and sulfadoxine in bacterial diseases of cattle and pigs. *Veterinary Record* **87**, 39.

Scholl, E., Bader, F., Campbell, D., Fluckiger, U., Gat, J., Glattli, H. R., Gonin, P., Hofer, A., Hilund-Carlsen, D., Meshorer, B. & Nesvadba, J. (1971). Veterinary trial of the chemotherapeutic preparation Borgal in infections of domestic animals in Switzerland, Denmark and Israel. *Schweizer Archiv für Tierheilkunde* **113**, 387.

Seboulet, A. (1971). Gonococcal or non-gonococcal urogenital infections. Results of treatment with sulphamethoxazole-trimethoprim. *Revue de Medecine* **23**, 1497.

Smith, H. W. & Halls, S. (1967). Observations by the ligated intestinal segment and oral inoculation methods on *Escherichia coli* infections in pigs, calves, lambs and rabbits. *Journal of Pathology and Bacteriology* **93**, 499.

Smith, H. W. & Linggood, M. A. (1972). Further observations on *Escherichia coli* enterotoxins with particular regard to those produced by atypical piglet strains and by calf and lamb strains: the transmissible nature of these enterotoxins and of a K antigen possessed by calf and lamb strains. *Journal of Medical Microbiology* **5**, 243.

Sojka, W. J. (1965). E. coli *in Domestic Animals and Poultry*. Commonwealth Agricultural Bureaux.

J. Hyg. Camb., (1973), **71**, 679
Printed in Great Britain

Diphthericin types, bacteriophage types and serotypes of *Corynebacterium diphtheriae* strains isolated in Australia

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(*Received* 5 *March* 1973)

SUMMARY

A diptheric in typing scheme has been constructed using 441 strains of *Corynebacterium diptheriae* isolated in eastern Australia from 1962 to 1971. Ten types have been distinguished using seven strains of *C. diphtheriae* and two strains of *C. belfanti* as indicators of the diphthericins produced by the newly isolated strains. Strains grouped into types L2, L3 and L3*a* were found only in Melbourne and types L1 and L4 were predominant in Sydney. Type L5 strains were isolated intermittently throughout the period of study and were found in all eastern states. Numerical analysis of the characteristics of the strains suggests that associations exist between, on the one hand, diphthericin type and, on the other hand, bacteriophage type, serotype and biochemical activity.

INTRODUCTION

The typing of *C. diphtheriae* by means of surface antigens (Ferris, 1950) or by sensitivity to bacteriophages (Saragea & Maximescu, 1966, 1969) has been in use for a number of years. It has been known for more than 20 years (Thibaut & Welsch, 1949; Thibaut & Fredericq, 1956) that *C. diphtheriae* produces bacteriocins and it was therefore decided to establish a typing scheme using these diphthericins. While the work was being carried out, independent reports of bacteriocin activity among strains of *C. diphtheriae* appeared from Russia (Emelyanov, Musonova & Lavnik, 1968) and Romania (Meitert, 1969). It was hoped that diphthericin typing would be useful with those strains which could not be typed by serological means or by their sensitivity to bacteriophages (Gibson, Cooper, Saragea & Maximescu, 1970).

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MATERIALS AND METHODS

Source of strains

The strains isolated in Victoria or received from New South Wales have been described previously (Gibson *et al.* 1970). South Australian strains were forwarded to the Microbiological Diagnostic Unit (M.D.U.) from the Institute of Medical and Veterinary Science in Adelaide, while the strains isolated in the Northern Territory, near Darwin, and those isolated in Cairns, Queensland, were received from the School of Public Health and Tropical Medicine in the University of Sydney.

Bacteriological techniques

The biochemical, serological, bacteriophage-typing and virulence tests were described previously (Gibson *et al.* 1970), but a few additions and amendments were made. The ability to liquefy gelatin was examined by growing the strain, for 14 days at 37° C., on a Loeffler slope upon which an Oxoid charcoal gelatin disk had been placed. The technique of Cook (1950) was employed for nitrate reduction using a strip of sterile filter paper, soaked with a 5 % solution of potassium nitrate, on horse blood agar. In this way six strains, together with one positive control strain of *C. diphtheriae* and one negative control strain of *C. belfanti*, could be tested on each plate.

Rabbit antisera containing glycerol were used for slide agglutination and, if a reaction was observed with any antiserum, the organism was retested in 76·0mm. × 13·0 mm., rounded-bottomed, glass test-tubes by adding an equal volume of a suspension of live organisms (equivalent to the 'Wellcome' opacity tube no. 2, Burroughs Wellcome and Co., London) to doubling dilutions of antiserum not containing glycerol. A titre of 1/160, or more, was recorded as significant.

In outline, the method of Gillies (1964), employed for *Shigella* strains, was used to determine the diphthericin activity of strains of *C. diphtheriae* and to determine the bacteriocin activity of other organisms in relation to the diphtheria bacillus. The strain being tested for production was inoculated as a thin diametric streak across freshly prepared Oxoid tryptone soya agar containing 5 % horse blood, and was incubated at 30° C., for 48 to 72 hr. The growth was scraped off and the remaining cells killed with chloroform vapour. A modification of the apparatus of Wahba & Lidwell (1963) was used for strains being tested for sensitivity. They were applied to the surface of the medium by a holder carrying ten stainless steel plates. Thin strips of filter paper, each soaked in a broth culture of a different strain, were placed in the grooves of a sterile templet into which the stainless steel plates fitted. After the edges of the stainless steel plates had been seeded, they were applied to the medium at right angles to the original line of growth. The cultures were incubated for 36 to 48 hr., at 30° C. or 37° C., and the results recorded.

RESULTS

Bacteriocin activity among the corynebacteria

The scheme of identification described by Cowan & Steel (1965) was followed for all strains of corynebacteria isolated but, in addition, strains of *C. belfanti* were distinguished from strains of *C. diphtheriae* by their failure to show definite reduction of nitrate, by their inability to kill guinea-pigs and by their failure to react with *C. diphtheriae* antisera. It should be mentioned that Gundersen (1959) observed serological cross-reactions between his strains of *C. belfanti* and *C. diphtheriae*. Strains of *C. ulcerans* were not encountered during the ten year period of study.

In general, amongst the corynebacteria tested, only *C. murium* and *C. hofmannii* produced bacteriocins reacting against *C. diphtheriae*, except those of *C. diphtheriae* itself. Bacteriocins of *C. diphtheriae* reacted against *C. belfanti* alone, as well as, of course, *C. diphtheriae*. Eighty-three strains of six species were tested for possible bacteriocin activity against 50 strains of *C. diphtheriae*. The latter strains were selected so that at least one representative of each serotype and of each bacteriophage type (or sensitivity pattern) found in Australia was examined. If the strains of *C. belfanti* (12 strains), *C. xerosis* (17 strains), *C. renale* (6 strains) and *C. bovis* (13 strains) produced bacteriocins then they were not active against our strains of *C. diphtheriae*. However, all five strains of *C. murium* were active against the three representatives of serotype Bennett and the four representatives of serotype Wagland used in these experiments. In addition, the 30 strains of *C. hofmannii*, isolated at the M.D.U., displayed two patterns of activity against the 50 diphtheria strains. The members of one group were active against all strains of *C. diphtheriae*, except those of serotype McLean, and the members of the other group were active against all diphtheria strains except those of serotype McLean and non-virulent strains of serotype 2 and serotype Nadjarian.

The same 50 strains of *C. diphtheriae* were tested for their production of bacteriocins (diphthericins) against the same 83 strains of corynebacteria. Activity was detected only against strains of *C. belfanti* and the pattern of activity is exemplified by the action against *C. belfanti* strains F3517 and F10157 (Table 1). Some strains of the diphtheria bacillus inhibited both strains of *C. belfanti*, some were active against one or other and some inhibited neither. These two strains of *C. belfanti* were therefore incorporated in the diphthericin typing scheme.

The diphthericin typing scheme

As a result of cross-testing 441 strains of *C. diphtheriae* and a few strains of *C. belfanti* isolated in Victoria, New South Wales, South Australia, Northern Territory and Queensland, since 1962, together with a few strains isolated previously, 10 diphthericin types have been distinguished (Table 1). Diphthericin production was chosen as the basis for the typing scheme, rather than diphthericin sensitivity, because the former system distinguished strains which behaved similarly when tested for sensitivity to these agents. Moreover, it seems that production is the more stable characteristic (Abbott & Shannon, 1958). Ten patterns of activity have been distinguished using nine indicator strains and comprise what can be

Table 1. *Diphthericin types of* C. diphtheriae *strains in Australia*

Producer type	Indicator (sensitive) strains*								
	Ferris† Nadjarian	Ferris Wagland	Ferris Bennett	F10157‡	F3517‡	1281§	201742‖	116888‖	Ferris McLean
L1	+ +	+ +	+ +	+ +	+ +	+ +	+ +	—	+ +
L2	—	+ +	+ +	+ +	+ +	+ +	+ +	—	—
L3	—	—	—	+ +	+ +	+ +	+ +	—	—
L3a	+ +	+ +	—	+ +	+ +	+ +	+ +	+ +	—
L4	—	—	—	—	+ +	+ +	—	—	—
L5	—	—	—	—	—	+ +	—	—	—
L6	—	—	—	—	+ +	—	—	—	—
L7	—	—	+ +	+ +	+ +	+ +	—	—	—
L8	+ +	+ +	+ +	+ +	—	—	+ +	—	+ +
LU	—	—	—	—	—	—	—	—	—

* + + Denotes growth inhibited; the diphthericin types of these strains are described in the text.
† Ferris (1950) stock serotypes.
‡ M. D. U. isolates identified as *C. belfanti*.
§ Strain of *C. diphtheriae* received from Dr Saragea.
‖ M. D. U. isolates of *C. diphtheriae*.

described as eight true types, one provisional type (L3a) and one type (LU) in which it is convenient to bring together those strains in which the ability to produce diphthericin was not demonstrated.

An example of type L1 is the indicator strain, M.D.U. strain no. 116888, and an example of type L2 is the Ferris (1950) stock strain of serotype Nadjarian. The indicator strains, Ferris (1950) stock serotypes Wagland and Bennett were generally inactive with respect to diphthericin production, although the former sometimes displayed activity against the indicator strain *C. diphtheriae* 1281 and the latter was occasionally active against *C. belfanti* strain F3517. The indicator diphtheria strain 1281 was received from Dr Alice Saragea and Dr Paula Maximescu and is the only strain which is sometimes capable of displaying activity against itself. All other isolates conformed to the rule that a producer strain was inactive against members of its own diphthericin type.

The indicator diphtheria strain 201742 was diphthericin type L5 and the Ferris (1950) stock strain of serotype McLean was diphthericin type LU. Moreover, the 27 strains which comprised type LU were not only all inactive with respect to diphthericin production, but were also resistant to the action of these agents including the bacteriocins of *C. murium* and *C. hofmannii*. Further, these strains were difficult to type by bacteriophage methods because they were either completely resistant to all bacteriophages in the set, or were sensitive only to bacteriophages 11 and 23 (bacteriophage type VI). The Ferris (1950) stock strain of serotype McLean was also sensitive only to bacteriophages 11 and 23 but differed from other strains of this diphthericin type and bacteriophage type since it was inhibited by the diphthericins produced by strains of diphthericin type L1 and the strain of diphthericin type L8.

Type L3a is provisional because the pattern of activity, shown in Table 1,

Table 2. *Distribution and occurrence of the diphthericin types in Australia*

Diphthericin type	No. of strains	Location	Year of isolation
L1	58	Sydney	1967–8
	15	Elmore and Swan Hill	1967
	1	Melbourne	1969
L2	98	Melbourne	1962–5, 1967, 1969
L3	15	Melbourne	1970
L3a	4	Melbourne	1970
L4	36	Sydney	1963–4, 1967, 1968
	9	Melbourne	1968, 1969, 1970
	3	Swan Hill	1967, 1968
	1	Lismore	1968
L5	91	Melbourne	1962–4, 1967–70
	33	Sydney	1963, 1967, 1968, 1971
	7	Swan Hill	1967
	6	Lismore	1968
	23	Adelaide	1969, 1970
	5	Darwin	1970
	7	Cairns	1972
L6	1	Melbourne	1970
L7	1*		
L8	1	Sydney	1971
LU	20	Melbourne	1964, 1971
	5	Lismore	1968
	2	Sydney	1971

* Hewitt (1947) strain of starch-fermenting serotype 1.

occurred after 24 hr. incubation but, after 72 hr., the pattern changed to that of type L3 since the growths of the indicator diphtheria strains Ferris Nadjarian, Ferris Wagland and 116888 had completely traversed the original diametric streak.

Occurrence of the diphthericin types in Australia

The distribution and occurrence of the diphthericin types in Australia is presented in Table 2. The majority of type L1 strains were detected in Sydney in the latter half of 1967 and throughout 1968; 15 strains were isolated in Central and Northern Victoria in mid-1967, but only one strain of this diphthericin type was found in Melbourne. In contrast, strains of type L2 were restricted to Melbourne and were isolated throughout the period between 1962 and 1965, in 1967 when one strain was found, and in November 1969 when 16 strains were isolated. Similarly, strains of types L3 and L3a were limited to Melbourne, but these were cultured during one period only, in January 1970, in the restricted environment of a mental hospital.

Type L4 strains were well established in New South Wales, in particular at Sydney and Lismore, and occurred in Sydney intermittently throughout the period of study. Type L5 strains were isolated in all eastern states of Australia and

have occurred at regular intervals during the 10 years, particularly in Melbourne and Sydney; approximately 40 % of the strains displayed this pattern of diphthericin production. An example of type L7 has not been isolated in Australia and only one isolate of each of the types L6 and L8 were obtained in Melbourne and Sydney respectively.

Comparison of diphthericin type with other typing characteristics

A numerical analysis of the characteristics of the strains was compiled by Miss Betty Laby and Professor E. J. Williams for Victorian isolates and was applied by the authors to include strains found in other eastern states of Australia. Comparison of diphthericin type with sensitivity to the bacteriophages numbered 1 to 24, with antigens detected at a titre of 1/160 or more, and with biochemical activity is presented in Table 3.

It can be seen that, in general, strains of a given diphthericin type fall into very few bacteriophage types and this is seen most strikingly with diphthericin type L2 in which 97 of 98 strains belonged to one bacteriophage type. However, not all strains of this diphthericin type were bacteriophage type XVI; one strain was sensitive only to bacteriophage 19 and the Ferris (1950) stock strain of serotype Nadjarian, classed as diphthericin type L2, exhibited the bacteriophage sensitivity pattern of type XIV similar to the Hewitt (1947) strain of serotype 1.

Resistance to bacteriophages 1–24 was common among diphthericin type L5 strains; 131 were completely resistant and all but one strain were sensitive to no more than four of the bacteriophages. This one strain, sensitive to bacteriophages 13, 14, 15, 16, 17 and 18, was the single strain of serotype Wallis obtained in 1964 and it is interesting that the Ferris (1950) stock strain of serotype Wallis and four strains of this serotype isolated in 1960 were also characterized as diphthericin type L5 and were sensitive to the same six bacteriophages.

Of the 74 diphthericin type L1 strains, 64 possessed the antigen 6387-Greenwood and five strains were autoagglutinable but were isolated during two outbreaks in which all the other strains were of serotype 6387-Greenwood. Ninety strains out of a total of 98 which were diphthericin type L2 possessed one or both of the antigens Nadjarian and 2 and, again, the five autoagglutinable strains were isolated from one outbreak involving strains of serotype Nadjarian. All isolates of diphthericin type L3 and the provisional type L3a were classed as serotype 6387-Greenwood. Of the 27 isolates of diphthericin type LU, one was serotype 6387-Greenwood, one was serotype Edmonston, 23 possessed antigen McLean and two were autoagglutinable but were associated with an outbreak in which strains of serotype McLean were isolated. As mentioned previously, the Ferris (1950) stock strain of this serotype was classed as diphthericin type LU.

Strains grouped into diphthericin types L4 and L5 possessed a wide range of antigens and two strains, isolated near Darwin, which were placed into type L5 produced even suspensions yet failed to react with any of the typing antisera. Although a wide range of antigens was found in types L4 and L5, it is notable that strains possessing the antigen Johnson were placed in one or other of these two diphthericin types.

Table 3. *Comparison of diphthericin type with other parameters used for typing* C. diphtheriae *strains isolated in Australia*

Diphthericin type	Bacteriophage type, or sensitivity	Antigenic* type	Biochemical† activity m	s	v	No. of strains isolated
L1	XIVC	6387-Greenwood	+	+	+	2
	XIVC	6387-Greenwood	+	−	+	48
	XIVC	6387-Greenwood	−	−	+	7
	8, 9, 10, 11.	6387-Greenwood, 2	+	+	+	1
	Resistant	6387-Greenwood, 2	−	−	+	6
	Resistant	McLean, 2	−	−	+	5
	Resistant	Untypable	−	−	+	5
L2	XVI	Nadjarian	+	+	+	56
	XVI	Nadjarian, 2	+	+	+	16
	XVI	2	+	+	+	4
	XVI	6387-Greenwood	+	+	+	3
	XVI	6387-Greenwood, 2	+	+	+	13
	XVI	Untypable	+	+	+	5
	19.	2	+	+	+	1
L3	XVI	6387-Greenwood	−	+	+	7
	3, 10.	6387-Greenwood	−	+	+	3
	Resistant	6387-Greenwood	−	+	+	5
L3a	Resistant	6387-Greenwood	−	+	+	3
	Resistant	6387-Greenwood	−	−	+	1
L4	XIVC	Nadjarian, Johnson	+	−	+	6
	XIVC	Nadjarian, Johnson	−	−	+	18
	XIVC	Nadjarian	−	−	+	9
	XIVC	Untypable	−	−	+	1
	XIVC	Untypable	+	−	+	1
	3, 10.	Johnson	−	−	+	2
	11.	Johnson	−	−	+	1
	11.	Johnson, 2	−	−	+	1
	Resistant	Johnson	−	−	+	1
	Resistant	Johnson, 2	−	−	+	1
	Resistant	6387-Greenwood	−	+	+	3
	Resistant	6387-Greenwood	−	−	+	2
	Resistant	6387-Greenwood, 2	−	−	+	1
	Resistant	Untypable	−	−	+	2
L5	IV	6387-Greenwood	−	−	+	11
	11, 18.	Johnson	+	−	+	3
	11, 13, 18, 19.	Untypable	−	−	+	6
	13, 14, 15, 16, 17, 18.	Wallis	+	−	−	1
	4, 5, 10.	Johnson	−	−	+	20
	Resistant	2, Johnson	−	−	−	2
	Resistant	2, 6387-Greenwood	−	−	−	1
	Resistant	2, Nadjarian	−	−	−	5
	Resistant	2, McLean	−	−	−	17
	Resistant	2, McLean	+	−	−	1
	Resistant	2	−	−	−	5
	Resistant	2	+	−	−	3
	Resistant	Johnson	+	−	+	21
	Resistant	Johnson	−	−	+	8

Table 3 (*cont.*)

Diphthericin type	Bacteriophage type, or sensitivity	Antigenic* type	Biochemical† activity m	s	v	No. of strains isolated
L5	Resistant	Nadjarian	+	−	+	9
	Resistant	Nadjarian	−	−	+	33
	Resistant	Nadjarian	−	−	−	4
	Resistant	Nadjarian, 2	+	−	+	3
	Resistant	Nadjarian, Johnson	+	−	+	1
	Resistant	Nadjarian, Johnson	−	−	+	1
	Resistant	Nadjarian, Johnson	−	−	−	1
	Resistant	6387-Greenwood	−	+	+	3
	Resistant	6387-Greenwood, 2	+	+	−	1
	Resistant	Untypable	−	−	+	7
	Resistant	Untypable‡	−	−	−	5
L6	Resistant	6387-Greenwood, 2	+	−	−	1
L7	XIV	1	+	+	+	1
L8	XIVC	6387-Greenwood	+	−	+	1
LU	VI	McLean	+	−	+	13
	Resistant	McLean	+	−	−	5
	Resistant	McLean	−	−	−	1
	Resistant	McLean, 2	−	−	+	4
	Resistant	6387-Greenwood	+	−	−	1
	Resistant	Edmonston	+	−	+	1
	Resistant	Untypable	+	−	−	2

* In those strains which carried two antigens, the antigen listed first was detected at a titre of 1/1280, or more; the other antigen was detected at a titre of 1/160 to 1/640. However, 10 of the 16 strains identified as diphthericin type L2 and serotype Nadjarian/2 reacted equally with both of the respective antisera to a titre of 1/1280, or more.

† m+ = acid from maltose; s+ = acid from starch; v+ = virulence for the guinea-pig.

‡ Even suspensions of two of these strains failed to react with any of the typing antisera. All other untypable strains were autoagglutinable in suspension.

The strains identified as *C. diphtheriae* were not homogeneous in the properties of maltose fermentation, starch hydrolysis and ability to kill guinea-pigs, and the non-random distribution of these properties between the different diphthericin types is detailed in Table 3. It is notable that the 56 non-virulent strains were confined to the types L5, L6 (one strain) and LU. Diphthericin type L2 was found to be composed of strains that fermented maltose and starch, and killed guinea-pigs. It was mentioned above that nearly all members of this diphthericin type also belonged to one bacteriophage type, yet the strains were isolated over a relatively long period of time (Table 2).

DISCUSSION

When the methods of typing *Corynebacterium diphtheriae* are compared, then 94 % of the strains isolated in eastern Australia were typed by the diphthericins they produced and, in the remaining 6 %, diphthericin production was not detected.

In comparison, 92 % of the isolates were typed by serological methods and, when the bacteriophages numbered 1 to 24 were used, 50 % of the strains fell into well-established bacteriophage types, a further 10 % displayed irregular patterns of sensitivity to these agents and the remaining 40 % could not be typed by the bacteriophages of this set, although a few strains were placed into two new types using bacteriophages in addition to those mentioned above (Gibson *et al.* 1970).

The strains which could not be typed on the basis of diphthericin production were grouped into a type which might be heterogeneous and it may be necessary to obtain strains from other countries in order to construct types for them. That this might be a fruitful line of work was indicated by strains grouped into diphthericin type L5 since they were distinguished by their action on *C. diphtheriae* strain 1281 received from Romania. At present type L5 appears widespread in Australia, but, as more indicator strains are found, it may be possible to subdivide it into further types. The existence of subtypes within diphthericin type L5 is indicated by the high degree of variation in biochemical activity, by the many bacteriophage types and by the many serotypes. However, one subtype might be composed of the 34 strains which were bacteriophage-resistant, did not ferment starch, were non-virulent and possessed major antigen 2; a second subtype might be composed of the 20 strains which were serotype Johnson and sensitive to bacteriophages 4, 5 and 10; and a third subtype might contain the 11 strains which were serotype 6387-Greenwood and bacteriophage type IV.

It is notable that diphthericin types L1 and L4 were found mainly in New South Wales and that the types L2, L3 and L3a were restricted to Melbourne. The epidemiological factors involved in the association of one diphthericin type with one location are not known, but another interesting facet of this study is that those New South Wales strains which were grouped into bacteriophage type XIVC (Gibson *et al.* 1970) were placed into either diphthericin type L1 or L4 and those Victorian strains classed as bacteriophage type XVI (previously called X–XVI–XVIII, Saragea & Maximescu, 1969) were all grouped into diphthericin type L2.

A second, little-understood group of associations emerging from this study are the relationships between diphthericin type and some of the other bacteriological characteristics used to identify the diphtheria bacillus. One well-documented 'classical' variety of *C. diphtheriae* possesses the ability to ferment maltose and starch, and is virulent for the guinea-pig. Nearly all such strains that were isolated came from Melbourne, belonged to diphthericin type L2, to the bacteriophage type XVI and varied only in the antigens possessed. The chronological appearance of these antigenic variants is interesting; strains with antigen Nadjarian appeared in 1962, 1963 and 1964, and, towards the end of 1964, strains serotyped as Nadjarian/2 were isolated. In March of the following year, the five serotype 2 strains were found. Two years later, a single strain of serotype Nadjarian/2 was obtained. Finally in 1969, one outbreak was caused by strains of serotype 6387-Greenwood/2 and strains of the highly specific serotype 6387-Greenwood. It is tempting to postulate that these strains are all descendants of a single,

parental strain which, during the 10-year-period, had undergone some form of 'antigenic drift'.

The strains which did not produce detectable amounts of diphthericin were virtually all of serotype McLean and those strains which were untypable by serological or bacteriophage methods were grouped into diphthericin type L5; hence, two or more independent methods of typing should be used in investigating the spread of strains in any outbreak of diphtheria.

Strains representative of some of the diphthericin types, serotypes and bacteriophage types described in this paper, together with the diphtheria indicator strains used for the diphthericin typing scheme, have been deposited with Professor McEntegart at the University of Sheffield, England, and the two indicator strains of *C. belfanti*, F10157 and F3517, have been deposited with the National Collection of Type Cultures, Colindale, London, being N.C.T.C. no. 10837 and N.C.T.C. no. 10838 respectively.

Miss Betty Laby and Professor E. J. Williams in the Department of Statistics, University of Melbourne, carried out numerical analyses presented in this paper. Thanks are given to Dr Alice Saragea and Dr Paula Maximescu for sending the bacteriophages of the typing scheme together with their propagating strains. A final word of appreciation is given to Mr Frank Anderson, in the School of Microbiology, for constructing the templet and the holder with stainless steel plates used for diphthericin typing.

REFERENCES

ABBOTT, J. D. & SHANNON, R. (1958). A method for typing *Shigella sonnei* using colicine production as a marker. *Journal of Clinical Pathology* **11**, 71.

COOK, G. T. (1950). A plate test for nitrate reduction. *Journal of Clinical Pathology* **3**, 359.

COWAN, S. T. & STEEL, K. J. (1965). *Manual for the Identification of Medical Bacteria*, p. 57. Cambridge University Press.

EMELYANOV, P. I., MUSONOVA, A. G. & LAVNIK, O. N. (1968). Possibility of determining bacteriocinogenic properties of diphtheria bacilli in epidemiological examination. *Zhurnal mikrobiologii, épidemiologii i immunobiologii* **45**, 143.

FERRIS, A. A. (1950). Serological types of *Corynebacterium diphtheriae* in Australia. *Journal of Pathology and Bacteriology* **62**, 165.

GIBSON, L. F., COOPER, G. N., SARAGEA, A. & MAXIMESCU, P. (1970). A bacteriological study of strains of *Corynebacterium diphtheriae* isolated in Victoria and New South Wales. *Medical Journal of Australia* i, 412.

GILLIES, R. R. (1964). Colicine production as an epidemiological marker of *Shigella sonnei*. *Journal of Hygiene* **62**, 1.

GUNDERSEN, W. B. (1959). Investigation on the serological relationships of *Corynebacterium diphtheriae*, type *mitis*, and *Corynebacterium belfanti*. *Acta pathologica et microbiologica scandinavica* **47**, 65.

HEWITT, L. F. (1947). Serological typing of *C. diphtheriae*. *British Journal of Experimental Pathology* **28**, 338.

MEITERT, E. (1969). Sur les bactériocines de *Corynebacterium diphtheriae*. II. Étude des bactériocines produites par *C. diphtheriae*, *C. ulcerans*, *C. atypique*, *C. hofmanni* et *C. xerose*. *Archives roumaines de pathologie expérimentale et de microbiologie* **28**, 1086.

SARAGEA, A. & MAXIMESCU, P. (1966). Phage typing of *Corynebacterium diphtheriae*. Incidence of *C. diphtheriae* phage types in different countries. *Bulletin of the World Health Organization* **35**, 618.

SARAGEA, A. & MAXIMESCU, P. (1969). Nouveaux types phagiques dans le schéma de lysotypie de *C. diphtheriae*. *Archives roumaines de pathologie expérimentale et de microbiologie* **28**, 1053.

THIBAUT, J. & FREDERICQ, P. (1956). Actions antibiotiques reciproques chez *Corynebacterium diphtheriae*. *Comptes rendus des séances de la Société de biologie* **150**, 1512.

THIBAUT, J. & WELSCH, M. (1949). Activité antibiotique de *Corynebacterium diphtheriae gravis*. *Comptes rendus des séances de la Société de biologie* **143**, 1002.

WAHBA, A. H. & LIDWELL, O. M. (1963). A simple apparatus for colicine typing. *Journal of Applied Bacteriology* **26**, 246.

J. Hyg. Camb., (1973), **71**, 691
Printed in Great Britain

Variation among strains of *Corynebacterium diphtheriae* during an outbreak in a restricted environment

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(*Received* 5 *March* 1973)

SUMMARY

Strains of *Corynebacterium diphtheriae* isolated from a small outbreak in the restricted environment of a Mental Hospital were examined. All belonged to one serotype, but there was marked variation in diphthericin type, in sensitivity to bacteriophages and in the minor antigens possessed. One strain was non-virulent and laboratory-produced variants of this non-virulent strain showed changes in some of the characteristics used in the identification and typing of the organism, such as diphthericin type, sensitivity to bacteriophages and diphthericins, virulence, starch fermentation and, to a lesser extent, in antigenic structure. The epidemiological and experimental findings are consistent with the hypothesis that the strains isolated, both in the hospital and in the laboratory, were derivatives of a single parent and the mechanism of some of the variations could be related to changes in some structural component such as the cell membrane or the cell wall.

INTRODUCTION

Bacteriological studies of strains of *Corynebacterium diphtheriae* isolated in Victoria and New South Wales demonstrated that a number of strains existed which did not conform to the 'classical' definition based on cultural, biochemical and serological characteristics (Gibson, Cooper, Saragea & Maximescu, 1970). It was argued that the large number of varieties found were derived from one parental type, but the mechanism of the variation was not understood although involvement of bacteriophage was considered possible.

In January 1970, an outbreak of diphtheria occurred in the restricted environment of a Mental Hospital and the strains isolated were examined for their cultural, biochemical and serological characteristics. In addition, they were studied for sensitivity to bacteriophages of the typing scheme and for their ability to produce diphthericins. During the course of diphthericin typing of these strains it was noticed that, on incubation of the culture plates for 5 days or more, colonies quite frequently developed within the zone of diphthericin activity when certain producer-indicator combinations were examined. It was decided, therefore, to test

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whether variants of a strain of *C. diphtheriae*, selected for insensitivity to the action of diphthericin produced by another strain, exhibited concomitant changes in other characteristics used in the identification and typing of the organism. This latter approach seemed especially relevant in view of the fact that pleiotropic changes had been observed in colicin-tolerant mutants of *Escherichia coli* (Holland *et al.* 1970; Onodera, Rolfe & Bernstein, 1970) and in aeruginocin-tolerant mutants of *Pseudomonas aeruginosa* (Holloway, Krishnapillai & Stanisich, 1971).

METHODS AND MATERIALS

Bacteriological techniques

The methods of examining strains of *C. diphtheriae* for their cultural and biochemical characteristics, antigenic reactions, bacteriophage sensitivity and virulence were described previously (Gibson *et al.* 1970). Since certain bacteriophages were not possessed by the author, some of the Mental Hospital strains were sent to Romania where they were tested by Dr Alice Saragea for their sensitivity to the bacteriophages numbered 25 to 35. Additional tests and the scheme for typing strains by their production of diphthericins were also described elsewhere (Gibson & Colman, 1973). Because of the large number of variants to be tested for virulence, the intradermal (Romer) method as described in *Medical Microbiology* (Cruickshank, 1965) was employed, but a few variants and all the Mental Hospital strains were tested by subcutaneous inoculation.

Isolation of diphthericin-insensitive variants

Producer strain and sensitive strain

The Ferris (1950) stock strain of serotype Nadjarian, designation FN, was used as the producer of diphthericin in these experiments. It differed in many characteristics (Table 2) from strain 201742 which was isolated from one of the Mental Hospital patients and was the parent of the diphthericin-insensitive variants (Tables 1 and 2).

Method of selection

An overnight broth culture of strain FN was used to inoculate Tryptone Soya Horse Blood Agar (T.S.H.B.A.) in glass Petri dishes. The inoculum was applied in a thin circle whose radius was approximately two-thirds that of the dish; in addition, the inoculum was applied to the centre of the circle. This technique ensured that the surface of the agar was thoroughly impregnated with diphthericin after incubation at 30° C., for 60 hr. The growth was removed and the remaining cells killed with chloroform vapour.

An overnight broth culture of strain 201742 was diluted 1/10 and 1/100; 0·1 ml. of each dilution and of the broth itself were spread over the surface of the diphthericin impregnated plates which, after drying, were incubated at 30° C., for 5–7 days. Colonies which developed on the plates were assumed to have arisen from cells which were insensitive to the diphthericin of strain FN and in one such

experiment the selection rate was 0·01 %. All colonies were plated on Potassium Tellurite Horse Blood Agar (P.T.H.B.A.) for purity. One colony from each P.T.H.B.A. plate was used to inoculate a Loeffler slope and the growth from the Loeffler slope was stored at −20° C., in 1·0 ml. horse serum (free of antibodies against diphtheria toxin and diphtheria bacilli) before their being re-tested for diphthericin insensitivity and other bacteriological characteristics.

Method of adsorption

The bacterial cells could be insensitive to the action of diphthericin by either of two mechanisms, namely tolerance and resistance, and these two states can be distinguished because tolerant cells will adsorb bacteriocin, but the resistant strains will not (Bhattacharyya, Wendt, Whitney & Silver, 1970). It was thus necessary to establish whether or not the derivative strains of 201742 would adsorb diphthericin, although they were insensitive to its action.

Attempts to induce the production of large amounts of diphthericin, in broth cultures of strain FN by ultraviolet irradiation or by mitomycin C, have not been successful and so a semi-quantitative adsorption experiment was performed. Strain FN was inoculated diametrically across T.S.H.B.A. in the manner used for diphthericin typing (Gibson & Colman, 1973). However, in order to standardize the method as much as possible, 20 ml. of medium was poured into glass dishes whose internal diameter was 8·8 cm. and depth was 1·6 cm. Strain FN was applied to the agar by means of a cylindrical swab stick whose diameter and length were 2·0 mm. and 8·6 cm. respectively. The swab stick was thoroughly seeded with culture and, after excess culture was allowed to drain, the stick was placed across the agar and then removed after 30 sec. In this way, the width of the growth of strain FN was always between 5·0 and 6·0 mm. After incubation at 30° C., for 48 hr., the growth was scraped off and the remaining cells killed with chloroform vapour. An overnight broth culture of adsorbing strain was spread across half of the T.S.H.B.A. at right angles to the original diametric streak. This culture was incubated at 41·5° C., for a further 48 hr., since preliminary experiments had shown that, although diphthericin produced at 30° C. is still active at the higher temperature, its actual formation, at 41·5° C., is inhibited.

After 48 hr. at 41·5° C., the growth was removed and the remaining cells were killed as before. The entire surface of the T.S.H.B.A. was then inoculated with 0·2 ml. of an overnight broth culture of the sensitive strain 201742. After overnight growth, at 30° C., or 37° C., the width of the zone of diphthericin activity was measured with calipers and the reduction in width, between that half of the plate which had been adsorbed and the other half of the plate which had not, was regarded as a measure of the amount of adsorption.

RESULTS

The mental hospital outbreak

Forty isolates were obtained from 16 cases and 19 apparently healthy carriers one of whom was bacteriologically diagnosed after a second swabbing and another

Table 1. *Characteristics of strains of* C. diphtheriae *isolated during an outbreak of diphtheria in a Mental Hospital*

No. of isolates	Diphthericin type	Bacteriophage* Type, or sensitivity	Minor† antigens	M.D.U.‡ strain no.
7	L3	XVI	–	.
3	L3	3, 10, 925/944, 951/950	–	.
3	L3	Resistant	–	.
1	L3	11, 12, 25, 26, 28, 34, 951/950	–	200790
1	L3	11, 12, 25, 26, 28, 34	–	201637
2	L3a	Resistant	–	.
1	L3a	11, 12, 25, 26, 28, 34	–	201642
1	L3a	11, 12, 25, 26, 28, 34	McLean	201389
4	L4	Resistant	–	.
1	L4	Resistant	McLean	.
1	L4	11, 12, 25, 26, 28, 34	2, McLean	200585
10	L5	IV	–	.
1	L5	IV	McLean	.
3	L5	Resistant	–	.
1§	L5	34, 35	2, McLean	201742

* Resistant signifies that the isolates were resistant to bacteriophages numbered 1–24, inclusive, and those agents numbered 925/944, 951/936, 951/939, 951/950 and 951/956.

† The serotype (major antigen) for all strains was 6387-Greenwood.

‡ The bacteriophage-sensitivity pattern of each of these strains was provided by Dr Alice Saragea.

§ This strain produced only a transient illness in the guinea-pig, i.e., non-virulent. The other 39 isolates were virulent.

Table 2. *Characteristics of the stock producer strain* FN, *the diphthericin-sensitive strain* 201742 *and the diphthericin-insensitive derivative groups*

Strain	Reaction to diphthericin of strain FN	Diphtheri-cin type	Bacterio-phage type or sensitivity	Major antigen (serotype)	Minor antigens	Colonial* appearance	Acid from starch	Virulence
FN (producer)	Insensitive	L2	XIV	Nadjarian	–	Daisy-head	+	+
201742† (wild type)	Sensitive	L5	34, 35‡	6387-Greenwood	2, McLean	Small daisy-head	–	–
201742FN/A (derivative)	Insensitive	L3	11, 12, 25,‡ 26, 28, 34	6387-Greenwood	–	Small daisy-head	–	+
201742FN/B (derivative)	Insensitive	L2	XIV	6387-Greenwood	2, McLean	Daisy-head	+	+

* For a description of the colloquial terms see Wilson & Miles (1964).

† Isolated during the Mental Hospital outbreak.

‡ Results from Dr Alice Saragea (personal communication, 1971).

only after the third attempt. Four of the cases were aged 21, 23, 29 and 31 respectively and the ages of the remaining cases ranged from 9–18 years. Six of the cases had received the complete primary course of three injections of 'triple antigen' within the first 4 years of life (three of the six had received the primary course plus one booster injection) and ten had not been given any prophylactic inoculation (personal communication from the Hospital Authorities, 1970).

Two isolations of *C. diphtheriae* were made on different days from three of the clinical cases. The first strain from the throat swab of one patient was characterized as diphthericin type L4, bacteriophage resistant and serotype 6387-Greenwood but the second isolation, made a few days later from a second swab, was diphthericin type L3, was sensitive to bacteriophages 3, 10, 925/944 and 951/950 yet was the same serotype as the first strain. In the second patient both strains were serotype 6387-Greenwood but the first was diphthericin type L5 and bacteriophage type IV whereas the second was diphthericin type L3a and was resistant to the bacteriophages of the typing system. Both strains from the third patient were serotype 6387-Greenwood and diphthericin type L3 but the first obtained was bacteriophage resistant while the second was bacteriophage type XVI.

The characteristics of all 40 strains are presented in Table 1. All strains were serotype 6387-Greenwood but five strains cross-reacted with antiserum McLean and two of them also cross-reacted with antiserum 2. Much variation was observed in their diphthericin types and more so in their sensitivity to bacteriophages. Eighteen strains were placed into two of the well-established bacteriophage types in that seven were grouped into type XVI, being sensitive to agents 10–24 inclusive, and eleven were sensitive to bacteriophages 7, 23 and 24 (type IV). Thirteen strains were bacteriophage resistant and the remaining nine exhibited patterns of sensitivity not yet recognized as specific bacteriophage types.

The strain 201742 differed from all the others in two characteristics; it did not kill the guinea-pig in the subcutaneous virulence test and it was sensitive to the diphthericins produced by those strains classed as diphthericin type L3 or type L3a.

The derivative groups

The bacteriological characteristics of producer strain FN, sensitive parent strain 201742 and the variants derived from strain 201742 are presented in Table 2. Variants selected for their insensitivity to the diphthericin of strain FN were placed in one of two 'derivative groups'. Strains of the group designated 201742 FN/A were selected at a rate of 1 in 10^4 and differed from the parent strain 201742 in four characteristics, namely diphthericin activity, bacteriophage sensitivity, serological reaction and virulence; whereas strain 201742 was capable of producing, in the guinea-pig, some of the pathological effects of diphtheria without actually killing the animal, the virulence of the derivative group 201742FN/A appeared fully restored.

The second group 201742FN/B was selected at a rate of 1 in 15×10^4. They were similar to the parent strain in their antigenic type, including serological cross-reactions, but they differed in their ability to ferment starch, in virulence, in the

Table 3. *Adsorption of diphthericin by strains of* C. diphtheriae

Adsorbing strain	Width of the zone of diphthericin activity (mm)		% reduction in zone size
	Before adsorption	After adsorption	
201742 (wild type)	23·0–25·0	13·0–15·0*	40·0
201742FN/A (derivative)	23·0–25·0	0·0	100·0
201742FN/B (derivative)	22·0–24·0	0·0	100·0
FN (producer)	23·0–25·0	0·0	100·0

* After two adsorptions with strain 201742, the zone of diphthericin activity was almost completely eliminated.

size of colony formed on P.T.H.B.A., in bacteriophage type and in diphthericin type. In fact, their characteristics were almost identical with those of the producer strain FN, but they were distinguished by their serological reaction since the producer strain was serotype Nadjarian.

A few colonies were selected from the impregnated T.S.H.B.A. plates which, on retesting for diphthericin insensitivity, were still sensitive to the diphthericin of strain FN. It was assumed that they had arisen from cells which had survived the various procedures taking place on the plate hence they were useful in that they served as controls in the subsequent bacteriological tests since they proved to be identical, in every characteristic, with the parent strain 201742.

Adsorption of diphthericin

Table 3 shows the results of adsorption using strain 201742, derivative strains 201742FN/A and 201742FN/B and producer strain FN as the adsorbing cultures. Using the indicator strain 201742 as the adsorbing strain, the width of the zone of diphthericin activity was 13·0–15·0 mm. in the region treated with adsorbing culture compared with a width of 23·0–25·0 mm. in that half of the plate which had not been inoculated with adsorbing culture. Thus, the zone of diphthericin activity was reduced by approximately 40 %.

Using the derivative strains 201742FN/A or 201742FN/B, or the producer strain FN for adsorption, diphthericin activity was completely eliminated since no lysis of the indicator culture was observed in the region where the adsorbing strain had grown, whereas in the other half of each plate a large zone of diphthericin activity was seen in the lawn culture. It is notable that the producer strain FN adsorbs its own diphthericin.

It is likely that the indicator strain 201742 achieved no more than 40 % reduction in zone size because the only cells available to adsorb diphthericin were those cells which constituted the inoculum, whereas the derivative strains and the producer strain were able to grow and multiply in the presence of diphthericin with the result that an ever increasing number of cells was available for adsorption. In

fact, if two adsorptions with strain 201742 were performed before adding the indicator lawn culture, the zone of diphthericin activity was almost completely eliminated.

The size of the zone of diphthericin activity appears to be a constant, reproducible characteristic for the producer strain/sensitive strain combination. Thus the zone of activity between producer strain FN and sensitive strain 1281 (Gibson & Colman, 1973) is always 35·0–37·0 mm., whereas the zone size formed between producer strain 201742FN/A and sensitive strain 201742 is 9·0–10·0 mm. It is notable that the zone size obtained with strain 201742FN/B as the producer and strain 201742 is the same size as the zone produced by strain FN and strain 201742. The 1·0–2·0 mm. range in zone size for a particular combination may be a reflection of the variation in width of the growth of the producer strain described in 'Method of Adsorption'.

DISCUSSION

The findings of Gibson & Colman (1973) indicated that an association existed between the diphthericin type of *C. diphtheriae* and other bacteriological characteristics used in the identification and typing of the organism. Even in the small outbreak which occurred in a Mental Hospital, some association was observed in that those strains which were more active in their production of diphthericins were, in general, sensitive to more bacteriophages, whereas of the 21 strains, which were relatively inactive in their diphthericin activity, eight were bacteriophage-resistant, 12 were sensitive to no more than three bacteriophages and only one was sensitive to six bacteriophages.

A more obvious feature of Table 1 is the wide variation in the characteristics of the strains isolated during an outbreak which occurred in the highly restricted environment of a hospital. Nineteen strains were diphthericin type L3 or L3a and exhibited five bacteriophage-sensitivity patterns; five were diphthericin type L4 and displayed two bacteriophage-sensitivity patterns; and the fifteen diphthericin type L5 strains showed three sensitivity patterns and one of them was non-virulent. The characteristic common to all strains was their serotype; the major antigen was 6387-Greenwood but five of the strains possessed antigen McLean and, in addition, two reacted with antiserum 2. The constancy of the major antigen was again apparent in the varieties derived from the Mental Hospital strain 201742; both derivative groups were serotype 6387-Greenwood, but the group 201742FN/A had lost minor antigens 2 and McLean. This group, however, displayed other characteristics similar to some of the Mental Hospital strains. For instance, 200585 possessed the same cross-reacting antigens and was sensitive to the same six bacteriophages. Again, derivative group 201742FN/A and strains 201389 and 201642 were sensitive to these six bacteriophages, and in addition, strain 201389 cross-reacted with antiserum McLean. Finally, the strain numbered 201637 was similar, in every characteristic examined, to this derivative group, being diphthericin type L3, serotype 6387-Greenwood and exhibiting the same bacteriophage sensitivity.

Two explanations can be offered for the variation of characteristics observed in

the Mental Hospital strains. The first is that there existed in the Hospital a number of varieties of the diphtheria bacillus, each of them acting independently in producing a similar disease. The second explanation is that the different forms or varieties described represented phases of a parent strain as it passed from host to host. The 'one-parent' concept seems the more likely explanation of both the epidemiological and experimental findings. Thus (*a*) wide variation in characteristics was found in strains isolated during a small outbreak which occurred in a highly restricted environment, yet the strains were related by their serotype; (*b*) two strains, again similar in serotype but differing in other characteristics, were found in the one individual; and (*c*) diphthericin-insensitive variants produced in the laboratory differed from the parent strain in some characteristics but retained their serotype antigen.

Although the mechanism of this variation is not understood, the simplest explanation for some of the differences observed, especially in the light of the variants derived experimentally, is that the derivatives had undergone alteration in some structural component such as the cell membrane or the cell wall. Such alterations explained the changes observed in colicin-tolerant mutants of *Escherichia coli* (Holland *et al.* 1970; Onodera *et al.* 1970) and aeruginocin-tolerant mutants of *Pseudomonas aeruginosa* (Holloway *et al.* 1971). Thus, the diphthericin-insensitive variants of *C. diphtheriae* strain 201742 were able to kill guinea-pigs and were sensitive to more bacteriophages. Changes in these characteristics could be associated in some way with the surface layers of the cell; for instance, toxic activity is correlated with a 'cord-factor' found at the cell surface (Kato, 1970) and the toxin itself may be synthesized at the cell membrane (Uchida & Yoneda, 1967); again, a substance which inhibits adsorption of bacteriophage appears to be derived from a surface component of the cell (Groman & McCormick, 1961).

It would be tempting to correlate the diphthericin-insensitive groups with the classes of mutants proposed by Bhattacharyya *et al.* (1970) for *Escherichia coli*, namely bacteriocin-tolerant and bacteriocin-resistant, but resistant (non-adsorbing) variants of *C. diphtheriae* were not isolated. However, a better understanding of the nature of diphthericin-insensitivity will be obtained when stable, high-titre preparations of diphthericin have been made in order to investigate their physical and chemical nature.

REFERENCES

BHATTACHARYYA, P., WENDT, L., WHITNEY, E. & SILVER, S. (1970). Colicin-tolerant mutants of *Escherichia coli*: resistance of membranes to colicin E 1. *Science, New York* **168**, 998.

CRUICKSHANK, R. (1965). *Medical Microbiology*, 11th ed., p. 181. Edinburgh and London: E. and S. Livingstone Ltd.

FERRIS, A. A. (1950). Serological types of *Corynebacterium diphtheriae* in Australia. *Journal of Pathology and Bacteriology* **62**, 165.

GIBSON, L. F. & COLMAN, G. (1973). Diphthericin types, bacteriophage types and serotypes of *Corynebacterium diphtheriae* strains isolated in Australia. *Journal of Hygiene* **71**, 679.

GIBSON, L. F., COOPER, G. N., SARAGEA, A. & MAXIMESCU, P. (1970). A bacteriological study of strains of *Corynebacterium diphtheriae* isolated in Victoria and New South Wales. *Medical Journal of Australia* i, 412.

GROMAN, N. B. & MCCORMICK, K. (1961). Relation between adsorption of diphtheria phage and its inactivation by an oleic acid activated inhibiter. *Journal of Bacteriology* **81**, 394.

HOLLAND, I. B., THRELFALL, E. J., HOLLAND, E. M., DARBY, V. & SAMSON, A. C. R. (1970). Mutants of *Escherichia coli* with altered surface properties which are refractory to colicin E2, sensitive to ultraviolet light and which can also show recombination deficiency, abortive growth to bacteriophage λ and filament formation. *Journal of General Microbiology* **62**, 371.

HOLLOWAY, B. W., KRISHNAPILLAI, V. & STANISICH, V. (1971). Pseudomonas genetics. *Annual Review of Genetics* **5**, 425.

KATO, M. (1970). Action of a toxic glycolipid of *Corynebacterium diphtheriae* on mitochondrial structure and function. *Journal of Bacteriology* **101**, 709.

ONODERA, K., ROLFE, B. & BERNSTEIN, A. (1970). Demonstration of missing membrane proteins in deletion mutants of *E. coli* K12. *Biochemical and Biophysical Research Communications* **39**, 969.

UCHIDA, T. & YONEDA, M. (1967). Evidence for the association of membrane with the site of toxin synthesis in *Corynebacterium diphtheriae*. *Biochemica et biopysica acta* **145**, 210.

WILSON, G. S. & MILES, A. A. (1964). *Topley and Wilson's Principles of Bacteriology and Immunity*, 5th ed., Vol. 1, p. 597. London: Edward Arnold Ltd.

J. Hyg., Camb. (1973), **71**, 701
Printed in Great Britain

The ecology of Whataroa virus, an alphavirus, in South Westland, New Zealand

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(*Received* 6 *March* 1973)

SUMMARY

The findings of a survey on the ecology of an alphavirus over the years 1964–9 are reviewed. Evidence is presented to show that wild birds constitute a vertebrate reservoir of the virus and that mosquitoes, primarily *Culiseta tonnoiri* and *Culex pervigilans*, which are both endemic New Zealand species, are responsible for summer transmission.

Serological evidence of infection was obtained in all years and evidence is presented to indicate that the virus is enzootic rather than being reintroduced each spring. The number of birds with antibody increased before mosquitoes became active in the spring and possible explanations of this are discussed.

The mean temperature in the hottest month in the study area is substantially below that in other areas with enzootic mosquito-borne viruses and experimental studies showed that Whataroa virus was able to replicate more rapidly in mosquitoes at low temperatures than any arboviruses previously studied.

The main natural focus of infection appeared to be in a modified habitat and the introduced song thrush (*Turdus philomelos*) to be the main vertebrate reservoir host.

INTRODUCTION

In New Zealand a number of rural summer epidemics of influenza-like disease suggestive of an arbovirus aetiology have occurred. Because of this a serological survey covering a variety of climatic and biological zones throughout the country was initiated in 1959–60. This led to identification of areas on the west coast of the South Island, where the proportion of fowl sera containing low-titre haem-agglutination-inhibiting antibodies against group A and group B arboviruses indicated the existence of an enzootic infection (Maguire & Miles, 1960). In the 1961–2 summer several strains of an alphavirus (group A arbovirus) were isolated from mosquitoes in an area of South Westland near the small township of Whataroa and in due course this virus was named 'Whataroa Virus' (Ross, Miles, Austin & Maguire, 1964; Maguire, Miles & Casals, 1967).

The extreme complexity of continental ecosystems makes the study of the ecology of an arbovirus extremely difficult and laborious. The existence of an enzootic alphavirus in N.Z. enabled us to make a study of a mosquito-transmitted virus in the relatively impoverished fauna of a large island group in a temperate climatic zone.

South Westland appeared to be the area with the largest amount of infection and a serological survey carried out there in 1963 revealed that at that time 21% of wild bird sera contained neutralizing antibody against Whataroa virus. Laboratory studies showed that when non-immune birds were infected subcutaneously they circulated high titres of virus in the blood for some days.

The variety of mammals in the area was small. They included the usual domestic animals together with large numbers of the introduced Australian brush-tailed possum (*Trichosurus vulpecula*). No other wild mammals were very numerous, but stoats (*Mustela erminea*) were present in considerable numbers and from time to time there was a sharp rise in the number of rats, either *Rattus rattus* or *R. norvegicus*, and occasionally sharp increases in the number of house mice (*Mus musculus*). In our attempts to trap house mice we did not find large numbers in the bush and only caught one mouse per 36 trap nights. We had no success in trapping rats in the area. The very high rainfall in this area (about 4800 mm. per year) is probably the reason for the lack of a stable population of small burrowing mammals and for the complete absence of rabbits (*Oryctolagus cuniculus*). A very few hares (*Lepus europaeus*) were seen from time to time and deer (mainly *Cervus elaphus*) are increasing in the area, but are not yet numerous. Chamois (*Rupicapra rupicapra*) are in large numbers high in the mountains, but only come down to level ground during winter months. We did not find antibodies in sheep or cattle in areas in which we had obtained positive avian and human bloods. The titre of antibody in the serum of trapped possums was low and, when they were inoculated subcutaneously with virus in the laboratory, we were unable to demonstrate viraemia. Similarly, laboratory rats showed very little viraemia when inoculated subcutaneously with this virus (Dempster, 1964). Evidence obtained from these preliminary studies strongly suggested that the main reservoir of this virus was in birds and this seemed the more likely since the related viruses, Sindbis virus and Western equine encephalitis virus, are both regarded as having their main reservoir in avian hosts.

Laboratory studies have indicated that a blood titre of 10^7 plaque forming units (p.f.u.) per ml. gave an ID 50 to engorging mosquitoes of the species tested (Austin, 1967). No titres of this order were found in any mammals other than suckling mice. Because of this we endeavoured to determine the role of different bird species in the ecology of Whataroa virus and studied birds in defined areas around the township of Whataroa at regular intervals over a period of 5 years. Certain other short term studies were made in special areas.

Study areas

The township of Whataroa is situated at lat. 43° 16′ S., long. 170° 22′ E., on the Whataroa flat, a river plain 16 km. long by 9 km. wide surrounded by forest-covered hills except for an outlet to the Tasman Sea (Fig. 1). Although much of it has been cleared for farming there are still very many large clumps of trees and areas of swamp where the hills merge into the plains. During the course of the study, birds were sampled regularly in four areas. These are described in detail elsewhere (Miles *et al.* 1971). They consisted of one area of rain forest, part of which

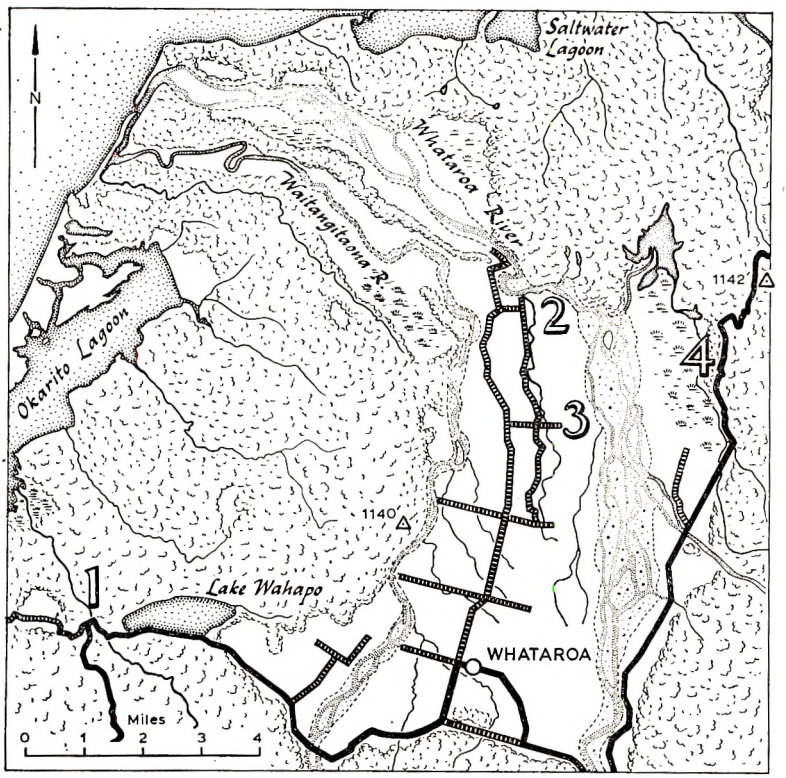

Fig. 1.

had been cut over and was in secondary growth, a fringe habitat between forest and swamp, an area of derelict swampy farm land close to forest and an area of improved land well away from the forest but including spinneys of thin bark totara (*Podocarpus hallii*).

Okarito Lagoon

A short-term survey was also made at Okarito lagoon, a coastal lagoon which carried a large permanent population of black swans (*Cygnus atratus*) and very substantial populations of migratory bar-tailed godwits (*Limosa lapponica*) in the summer and of other water birds. This area was abandoned quite quickly because few birds had antibody and the population of vectors was small and therefore it did not appear to be of any great significance in the ecology of our virus.

MATERIALS AND METHODS

Birds

Birds were captured in mist nets, banded, bled from the jugular vein and released. The volume of blood taken varied from 0·05 to 0·4 ml. according to the size of the bird. Blood samples were diluted immediately to a total volume of 1 ml. and placed in a chilled insulated container. They were frozen to $-70°$ C. within a few hours of collection and kept frozen until tested. Other methods of trapping were

sometimes used and, during the open season, duck shooters were supplied with sterile bottles and asked to collect samples of blood from birds which they shot. Little reliance could be placed on the results obtained with these last specimens.

Diluent

Ten per cent calf serum in Hanks's balanced salt solution containing 10 units of heparin and 100 units of penicillin and streptomycin per ml. was used.

Neutralization

Plasma samples were tested for neutralizing antibodies by a plaque reduction test on monolayers of duck or chick embryo cells by the method described by Ross *et al.* (1964). The samples of blood were screened at the dilution at which they were collected and those containing virus neutralising antibody were titrated. Samples which neutralized at dilutions of 1:10 or greater were regarded as positive. When birds were sampled more than once a fourfold or greater change in antibody titre was regarded as a significant change.

RESULTS

The main investigation was continued over a period of 5 years from February 1964 to February 1969. Eight bird collections were made during the first 14 months, then from April 1965 till the project was concluded birds were collected and bled at approximately monthly intervals. The average number of plasmas from each collection tested was 90. During the 5 years in which birds were sampled, 4554 bird plasmas from 30 species were tested for Whataroa virus plaque neutralizing antibodies. Of these, 673 (15%) had significant levels of antibody. In the analysis considered here the collections were divided into 12-monthly periods starting at the beginning of spring on 1 August and finishing 31 July. In this analysis the plasma from birds caught before 1 August 1964 have not been included. Fig. 2 shows the proportion of positive plasmas in all areas together in each season. The high proportion of birds circulating antibody in the 1965–6 season suggests the occurrence of a Whataroa virus epizootic. During that period, birds were being caught at three sites (1, 2 and 3 in Fig. 1). The increased incidence of immune birds was observed in all areas.

Table 1 shows the incidence of immunity in each of six species most commonly caught in the area plus the pooled incidence in other species. It was found that each season about 20% of song thrushes (*Turdus philomelos*) were circulating Whataroa virus-neutralizing antibody with little variation from year to year. The proportion of blackbirds (*T. merula*) with antibody was the same as that in thrushes in 1964–5, but there was a steady decrease over the 5 year period. All other species showed the same pattern of low incidence in the 1964–5 season followed by a sharp increase in 1965–6 and then a decline to the previous low levels in the following seasons.

The variation in the proportion of positives at different times of year is of interest. In the epizootic season (1965–6) 34% of the birds tested between February and July were blackbirds and thrushes, but they included only 13% of the positive plasmas. In this season the proportion of positive plasmas increased more or less

Table 1. *Yearly incidence of Whataroa virus neutralizing antibody in individual bird species*

Species	1964–5		1965–6		1966–7		1967–8		1968–9	
	No.*	% + ve	No.	% + ve	No.	% + ve	No.	% + ve	No.	% + ve
Thrush (*Turdus philomelos*)	10/49	20	35/152	23	36/225	16	23/117	20	7/41	17
Blackbird (*T. merula*)	12/60	20	34/283	12	39/295	13	7/153	5	0/43	0
Silver-eye (*Zosterops lateralis*)	2/136	2	189/299	63	42/441	10	4/524	1	2/89	2
Chaffinch (*Fringilla coelebs*)	4/59	7	54/88	61	12/85	14	0/70	0	0/31	0
Hedge sparrow (*Prunella modularis*)	0/18	0	46/77	60	7/111	6	1/58	2	1/27	4
Bellbird (*Anthornis melanura*)	3/20	15	15/40	38	4/57	7	0/22	0	0/12	0
Other species	1/90	1	67/156	44	10/238	4	2/145	1	0/56	0

* Number with antibody/number tested.

steadily from November through to the following March, when about 70% of all sera contained antibody. The findings in the enzootic seasons were quite different. The incidence of immune birds reached a peak in the early spring (September) followed by a drop in October and a second peak in November and December. Thereafter the incidence dropped to a very low level between February and July. During this period, 15% of the plasmas were from blackbirds and thrushes, yet these two species accounted for 50% of the positive plasmas.

On 540 occasions, birds were recaptured and bled after intervals from less than 1 month to 52 months. The antibody titre increased between bleedings on 43 occasions and decreased on 34 occasions. Eight birds had the same antibody titre in two plasma samples. The remaining 455 birds were free from antibody at both first and second bleedings.

All 43 birds which showed an antibody increase were exposed for all or part of the period from September to March. A drop in antibody titre was recorded after periods ranging from 2 to 3 weeks to 31 months. The birds which maintained their antibody titres were recovered after intervals ranging from 1 to 17 months (Table 2).

Mosquitoes

In the areas in which we have worked, two species of mosquitoes were dominant, while others occurred in special localities. In the rain forest, a large culicine *Culiseta tonnoiri* is the main mosquito, while in more open country the dominant is *Culex pervigilans*. In the fringe habitats in which we have been particularly interested and from which we obtained our virus isolations, both these species were present in proportions varying with the area, the rainfall in the recent past and the time of year. In the coastal areas they are replaced by the halophilic *Aedes australis*.

Table 2. *Changes in antibody titres of recaptured birds*

Month of first sample	No. of birds showing a change in antibody titre between samples*			
	Increase	Unchanged	Decrease	Total
August, September, October	16 (73 %)	0	6 (27 %)	22
November, December, January	17 (49 %)	5 (14 %)	13 (37 %)	35
February, March, April	5 (33 %)	3 (20 %)	7 (47 %)	15
May, June, July	5 (38 %)	0	8 (62 %)	13

* Plasma samples of 455 birds which were free of antibody on both occasions have been omitted.

Our isolations were made from *Culiseta* and *Culex* species, but owing to the difficulties of establishing them in our insectory, most of our studies on virus replication in mosquitoes were made on *Ae. australis*.

In our area the January isotherm is 16° C. and none of the mosquitoes of southern New Zealand will survive for a substantial period at 25° C. Our insectory was held at approximately 20° C., the highest temperature which allowed satisfactory survival of our mosquitoes.

Mosquitoes were allowed to feed on viraemic suckling mice and were then killed after varying intervals. The organs were disrupted by sonication and titrated by a plaque technique on chick-embryo monolayers. In 25 % of mosquitoes, virus leaked into the haemolymph within a few hours of feeding. In a few (about 10 %), substantial amounts of virus leaked out, probably because of damage of the wall of the intestine (Miles, Pillai & Maguire, 1973).

Apart from this, up to the 6th day, virus could only be demonstrated in the intestine. On the 6th day, 2 of 8 mosquitoes showed virus generalized through all their organs. In one of these the virus titre throughout was very high. Probably this was the result of a substantial early leak of virus into the haemolymph such as has been described above. Thereafter the proportion of mosquitoes with generalized virus increased until on the 17th day virus had generalized in 13 of 16 tested and these carried substantial amounts in the salivary glands. Although virus could be demonstrated in the salivary glands of most mosquitoes by the 8th day, only 1 of 31 tested for infectivity on day 14 succeeded in infecting a suckling mouse. However, on day 17, 6 of 10 mosquitoes which engorged on suckling mice transmitted infection. The results indicate that at least 10^4 plaque forming units (p.f.u.) of virus must be contained in the salivary glands for transmission to take place.

Table 3. *Multiplication of Whataroa virus in* Ae. australis *engorged on viraemic suckling mice*

Time after engorgement	Proportion of mosquitoes with virus in haemolymph	Proportion of mosquitoes with generalized virus in organs	Proportion of mosquitoes with $>10^4$ p.f.u. virus in salivary glands	Proportion of mosquitoes transmitting infection to suckling mice
30 min.	*3/10	—	—	—
4 hr.	1/7	—	—	—
1 day	2/7	—	—	—
2 days	3/10	—	—	—
5 days	—	0/8	0/8	—
7 days	—	2/8	0/8	—
9 days	—	7/12	1/12	—
11 days	—	8/11	1/11	—
14 days	13/16	7/10	1/10	1/31
17 days	4/8	13/16	10/14	6/10

— = Not tested.
* Number with specified infection/number tested.

This was only consistently present in mosquitoes with generalized virus which had been infected for 17 days, the longest period tested (Table 3).

After more success had been obtained in keeping *C. tonnoiri* in the insectory a limited number of experiments was carried out which showed that the susceptibility of this species was similar to that of *Ae. australis* and that multiplication of the virus in the two species at 20° C. was not greatly different.

The other most common haemophagous insect in the area is a simuliid, *Austrosimulium ungulatum*. Austin (1967) carried out a series of experiments on the vector potential of this species. He found that virus injected into the haemocoele would replicate, but that no concentration in the salivary glands took place. Further the insects could not be infected by feeding, but could transmit virus mechanically during 24–48 hr. after feeding on a viraemic suckling mouse. Thus this species might be able to transmit virus mechanically at the peak of an epidemic when many birds are viraemic, but could not be the main vector under normal enzootic conditions.

Feeding preferences

The available information on feeding preferences of New Zealand mosquitoes is limited. We know from experience that man is attractive to all species. Horses make excellent bait and chickens and rabbits attract approximately the same number of mosquitoes when used in the same trap on alternate nights. Apparently all species in the study area are indifferent feeders. They appear to be equally ready to bite birds and mammals.

Viraemia in birds

A series of experiments has been carried out to find the approximate ID50 for some species of birds which may be ecologically important for Whataroa virus, and

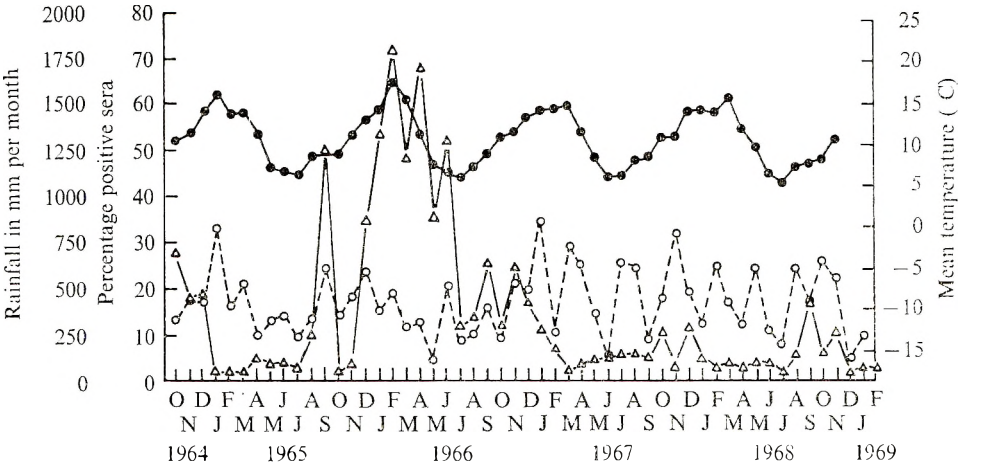

Fig. 2. Mean temperatures and rainfall and percentage of captured birds with antibody. ●, Mean temperature (°C); ○, total rainfall (mm/month); △, percentage of birds with neutralizing antibody.

to find the extent and duration of viraemia. The birds were collected in an area where there is good evidence that Whataroa virus circulates rarely if at all, and none possessed antibody before infection. The ID 50 for the three species for which an estimate was possible was between $10^{3\cdot0}$ and $10^{3\cdot3}$ p.f.u. of virus. This requirement of quite a substantial dose of virus before regular infection occurs probably explains why, although a high proportion of mosquitoes have virus in their salivary glands from the 8th day on, regular transmission is not obtained until 17th day. The maximum viraemia found varied from $10^{7\cdot1}$ to $10^{8\cdot2}$ p.f.u./ml. (F. J. Austin, in preparation). It has been estimated for some species of mosquito that the average amount of blood taken at a feed is about 0·003 ml. Our figures are not greatly different from this. Therefore a high proportion of mosquitoes feeding on birds with this level of viraemia would be expected to become infected.

DISCUSSION

The information obtained from this longitudinal survey of Whataroa virus infection of wild birds in South Westland indicates that the virus was present in the area throughout the study period. For most of the time the incidence of wild-bird plasmas with Whataroa virus-neutralizing antibody was less than 25%, suggesting an enzootic situation. In September 1965 the proportion of plasmas with antibody rose sharply and remained above 30% almost continuously until June the following year. Since there was no evidence of illness or death among the birds this may be regarded as a clinically inapparent epizootic situation.

The reasons for the occurrence of the epizootic in 1965–6 are obscure. The total annual rainfall of 4200 mm. was somewhat below that in other years of the study and the mean January temperature at 17° C. slightly above, but the climatic conditions at the time of the first antibody peak in September 1965 were in no way abnormal and the conditions in the late summer when the number of birds showing evidence of infection was very high were in no way different from those in the previous year when the proportion of birds with antibody was very low (Fig. 2).

During the enzootic years the highest proportion of immune birds occurred in the spring or early summer. There was an initial peak in September followed by a drop in October and a second peak in November or December. This was succeeded by a steady decline in frequency of antibody carriers to very low levels during the late summer, autumn and winter. Further evidence for the major spread of Whataroa virus in the spring and early summer is provided by the recovery data. All of the birds which developed antibody between captures were exposed for all or part of the period between September and March and presumably were infected during that time or immediately before their first capture. Those birds which showed a decrease in antibody between bleedings must have been infected before they were first bled. A similar decrease in antibody has been observed by South African workers who, on the basis of their field observations and experimental studies, concluded that some birds responded to Sindbis virus infection with only a transient antibody response (McIntosh, McGillivray, Dickinson & Taljaard, 1968; McIntosh, Dickinson & McGillivray, 1969; McIntosh, Madsen & Dickinson, 1969).

This pattern of high antibody incidence in birds in the early summer followed by a decrease about midsummer differs from that reported by workers who have studied the activity of other mosquito-borne alphaviruses in wild birds. Reeves & Hammon (1962) showed that in California the incidence of Western equine encephalitis (WEE) virus antibodies in house finches and English sparrows remained at summer levels at least until September. In a study in Alabama covering a period of 14 months Stamm (1968) showed that the highest incidence of both Western and Eastern equine encephalitis antibodies was found in wild birds bled in the late summer and winter and that the lowest frequencies occurred in midsummer.

Our pattern may be affected by the migration of birds from the mountain areas to the plains at the end of the breeding season. During the months of August to January the average number of silver-eyes captured was 3·4 per day and the proportion of birds netted which had been previously captured was 20%. These figures clearly indicate a resident population. From February to April the captures were 12·1 per day and the recovery rate fell to 3·3%. The introduced finches show a similar pattern. Assuming that these unbanded birds came from higher ground where the virus was not enzootic they would be expected to dilute the proportion of positive sera greatly except when virus circulation was so intense that they would also rapidly become infected as in the epizootic of 1965–6.

All the evidence we have obtained indicates that Whataroa virus exists in a bird–mosquito cycle, but the early spring rise in the proportion of birds with serum neutralizing antibodies is difficult to interpret on the basis of spread by mosquitoes. These are inactive in the winter and early spring and, although they have been observed to engorge indoors in the warm as early as July, this has not been seen in the open air. Substantial numbers of mosquitoes did not appear before October.

The possibility of some other vector has been considered. Studies carried out in the United States in the early 1950s make it seem improbable that bird mites are importantly involved, and a limited study failed to reveal infected mites in nests or viraemia in nestling thrushes (F. J. Austin & J. A. R. Miles, unpublished). Ticks

have only been found in the coastal areas in Westland and none have been proved to be natural vectors of alphaviruses, although Whataroa virus can replicate in *Ornithodoros capensis* (Ross, 1971). Austin's studies have shown that the only simuliid in the area can carry Whataroa virus mechanically, but not biologically. It would therefore only be likely to be effective in transmitting this virus at the peak of a very intense epizootic.

The annual reintroduction of the virus by migrating birds seems improbable. There are only two migrant species commonly seen inland in the study area. These are the shining cuckoo (*Chalcites lucidus*) and the long-tailed cuckoo (*Eudynamis taitensis*). These arrive too late in the season to explain the rise in the number of sero-positive birds in September. Bar-tailed godwits (*Limosa lapponica*) begin to arrive in mid-August and reach substantial numbers in September. However, they are only found on the coast and around coastal lagoons where they are relatively little exposed to mosquitoes, although very exposed to simuliids. They are unlikely to be responsible for a regular reintroduction of virus. The one trans-Tasman migrant, the double banded dotterel (*Charadrius bicinctus*), is rare in South Westland and could not be significant in the virus cycle.

We did not succeed in devising a technique for trapping godwits in our area, but we did make a short-term study of water birds around Okarito Lagoon where godwits were most numerous. Duck and swan bloods obtained from shooters in the 1963 winter had given a high proportion of positive virus neutralization tests. These results were suspect because the trauma of shooting often leads to release of non-specific virus-neutralizing substances into bird blood (Scherer, Hardy, Gresser & McClure, 1964). In the late summer of 1964 when the black swans (*Cygnus atratus*) were moulting we captured approximately 100 and obtained blood specimens from the wing veins. Only two gave positive tests. In the following summer none of 12 silver gulls (*Larus novaehollandiae*) and few swans had virus-neutralizing antibody. If godwits were important in the introducing of virus each year, one would expect a higher infection rate in birds using the same feeding grounds.

We are inclined to favour an alternative view that the endocrine changes and other physiological stress at the beginning of the breeding season lead to a recrudescence of a latent infection and thus to stimulation of antibody production. Our failure to demonstrate any evidence of sero-conversions from negative to positive between April and August and the observation of birds with the same antibody titre in plasma samples taken up to 17 months apart encourage us to think that latent infections may be important in the enzootic survival of virus in the area. We have no direct evidence as yet, either field or experimental, for or against this theory.

In the epizootic season the proportion of positive sera continued to rise until the end of the summer when about 70% of all bird plasmas contained significant antibody titres. This is the same scale of involvement as that reported by Stamm (1963), who observed that at the end of an arbovirus transmission season up to 70% of the birds in a local population may be circulating virus-neutralizing antibodies. Although bird plasmas and mosquito extracts collected during this period

were inoculated into suckling mice and onto duck embryo cell cultures, no arbovirus isolations were made. This is surprising considering the high infection rate of birds, but is probably due to the poor conditions under which the specimens were sometimes held in the field.

In the first enzootic season 20% of the thrushes and blackbirds were circulating Whataroa antibodies, but few of the other birds were. In the following season there was a considerable increase in the number of birds with antibody. The increase occurred in all species except thrushes and blackbirds. Subsequently the antibody rate decreased for all species except thrushes and in the last season of the survey *T. philomelos* was the only species in which significant numbers of immune birds had been detected. In a small series collected early in October 1972 a high proportion of thrushes was again shown to carry neutralizing antibody against the virus (J. A. R. Miles & F. J. Austin, unpublished).

Because of these serological findings we think that it is probable that *Turdus* species are important for the maintenance of the virus in the area. If this is correct it is interesting that a virus showing distinct differences from related viruses in other countries should be maintained in a reservoir dominated by species introduced into New Zealand only about 100 years ago. However, the areas in which we have evidence that the virus is enzootic are habitats modified by man in such a way as to give a considerable advantage to introduced birds of fringe habitats and farm lands. Our findings demonstrate that a new cycle of a mosquito-borne arbovirus can become established in such a modified habitat using introduced species as reservoir hosts. The unmodified habitats which were studied did not appear to contain a similar natural enzootic focus, but were only involved in epizootic situations.

Mosquitoes

The effects of temperature on the cycle of Japanese encephalitis virus in mosquitoes have been studied and Huang (1957) found that in *Culex pipiens* only 3 of 21 mosquitoes held at 18–22° C. became infectious compared with 17 of 20 kept at 31° C., a temperature similar to that found in the epidemic season in China and Japan. Hess, Cherubin & La Motte (1963) reported that St Louis virus, related to Japanese encephalitis virus, had similar temperature requirements, but that WEE virus became epidemic at rather lower ambient temperatures. However, the mean temperatures in the areas where WEE is epidemic are not below 21° C. in the epidemic season. Chamberlain & Sudia (1955) found that for another alphavirus, eastern equine encephalitis virus, in *Ae. triseriatus* reducing the temperature from 26·5 to 21° C. increased the time for virus to reach peak transmissibility from 17 to 34 days.

The mean temperature in the hottest month in our study area of 16° C. is lower than that of any other area with an enzootic mosquito-borne virus of which we have heard. Also the extrinsic incubation time of 17 days at 20° C. in *Ae. australis* and a minimum extrinsic incubation in *C. tonnoiri* of 10 days at the same temperature indicates a much more efficient replication in the insect host at low temperatures than in other systems which have been reported.

These findings suggest a high degree of adaptation in this virus to replication in mosquitoes at the relatively low temperatures found in our study area and indicate that strains of virus have been enzootic there for a considerable period. It is then the more interesting that all the evidence points to the main natural focus of this virus being now in a modified habitat with one or two passerine bird species introduced into the area not more than 100 years ago as the main vertebrate reservoir.

This work was mainly supported by the Medical Research Council of New Zealand and assisted by a grant from the Golden Kiwi Lottery Medical Research Distribution Committee. My thanks are also due to the Editor of the *Australian Journal of Experimental Biology and Medical Science* for permission to reproduce Fig. 1 and Table 2, and to the Editor of the *Journal of Medical Entomology* for permission to reproduce Fig. 2.

REFERENCES

AUSTIN, F. J. (1967). The arbovirus vector potential of a simuliid. *Annals of Tropical Medicine and Parasitology* **61**, 189–99.

CHAMBLERAIN, R. W. & SUDIA, W. D. (1955). The effects of temperature upon the extrinsic incubation of Eastern equine encephalitis in mosquitoes. *American Journal of Hygiene* **62**, 295–305.

DEMPSTER, A. G. (1964). *Trichosurus vulpecula* and *Rattus norvegicus* in the epidemiology of two arboviruses. B.Med.Sci. Thesis, University of Otago.

HESS, A. D., CHERUBIN, C. E. & LA MOTTE, L. C. (1963). Relation of temperature to activity of Western and St Louis encephalitis viruses. *American Journal of Tropical Medicine and Hygiene* **12**, 657–67.

HUANG, C. H. (1957). Studies of virus factors as causes of inapparent infection in Japanese B encephalitis: virus strains, viraemia, stability to heat and infective dosage. *Acta Virologica* **1**, 36–45.

MCINTOSH, B. M., MCGILLIVRAY, G. M., DICKINSON, D. B. & TALJAARD, J. J. (1968). Ecological studies on Sindbis and West Nile viruses in South Africa. IV. Infection in a wild avian population. *South African Journal of Medical Science* **33**, 105–12.

MCINTOSH, B. M., DICKINSON, D. B. & MCGILLIVRAY, G. M. (1969). Ecological studies on Sindbis and West Nile viruses in South Africa. V. The response of birds to inoculation of virus. *South African Journal of Medical Science* **34**, 77–82.

MCINTOSH, B. M., MADSEN, W. & DICKINSON, D. B. (1969). Ecological studies on Sindbis and West Nile viruses in South Africa. VI. The antibody response of wild birds. *South African Journal of Medical Science* **34**, 83–91.

MAGUIRE, T. & MILES, J. A. R. (1960). Evidence of infection with arthropod-borne viruses in New Zealand. *Proceedings of the University of Otago Medical School* **38**, 25–7.

MAGUIRE, T., MILES, J. A. R. & CASALS, J. (1967). Whataroa virus, a group A arbovirus isolated in South Westland, New Zealand. *American Journal of Tropical Medicine and Hygiene* **16**, 371–3.

MILES, J. A. R., PILLAI, J. S. & MAGUIRE, T. (1973). The multiplication of Whataroa virus in mosquitoes. *Journal of Medical Entomology*.

MILES, J. A. R., ROSS, R. W., AUSTIN, F. J., MAGUIRE, T., MACNAMARA, F. N. & ROSS, LESLEY M. (1971). Infection of wild birds with Whataroa virus in South Westland, New Zealand. *Australian Journal of Experimental Biology and Medical Science* **49**, 365–76.

REEVES, W. C., & HAMMON, W. MCD. (1962). In *Epidemiology of the Arthropod-borne Viral Encephalitides in Kern County, California* 1943–52, p. 223. University of California, Berkeley.

ROSS, R. W. (1971). The survival of Whataroa virus in New Zealand ticks, *Ornithodoros capensis* (Neumann) Argasidae: Acarinae. *Proceedings of the University of Otago Medical School* **49**, 38–40.

Ross, R. W., Miles, J. A. R., Austin, F. J. & Maguire, T. (1964). Investigations into the ecology of a group A arbovirus in Westland, New Zealand. *Australian Journal of Experimental Biology and Medical Science* **42**, 689–702.

Scherer, W. F., Hardy, J. L., Gresser, I. & McClure, H. E. (1964). Arbovirus neutralising substances in avian plasmas. I. Their higher prevalences after collection of birds by shooting and cardiac puncture than after netting and venipuncture. *American Journal of Tropical Medicine and Hygiene* **13**, 859–66.

Stamm, D. D. (1963). Susceptibility of bird populations to Eastern, Western and St Louis encephalitis viruses. *Proceedings 13th International Ornithological Congress* **1**, 591–603.

Stamm, D. D. (1968). Arbovirus studies in birds in South Alabama. *American Journal of Epidemiology* **87**, 127–37.

J. Hyg., Camb. (1973), **71**, 715
Printed in Great Britain

Two community outbreaks of human infection with *Yersinia enterocolitica*

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(*Received* 6 *March* 1973)

SUMMARY

Two outbreaks of human infection with *Yersinia enterocolitica* in Shizuoka, Japan are described. This is the first report of community outbreaks of infection with this organism in Japan, and possibly in the world. All the strains isolated in each outbreak belonged to O antigen group 3, biotype 4, of the species. Despite much effort, the source and mode of spread of the infection were not established.

INTRODUCTION

Although sporadic cases of human infection with *Yersinia enterocolitica* have been reported in western countries (Niléhn, Sjöström, Damgaard & Kindmark, 1968; Winblad, Niléhn & Sternby, 1966) and recently in Japan (Zen-Yoji & Maruyama, 1972), community outbreaks of infection have not previously been described.

Epidemics of *Y. enterocolitica* infection occurred among children in two separate communities in the Shizuoka Prefecture, Japan. These outbreaks were investigated by a team from the Shizuoka Department of Public Health and Public Health Laboratory; the organisms isolated were studied at the National Institute of Health, Tokyo. The results of these studies are presented in this paper.

MATERIAL AND METHODS

Stool specimens

Specimens were collected not only from patients but also from unaffected individuals in the same community or families as the patients. Additional stool specimens were obtained from staff working in a food catering institution which provided lunches for the community, as well as from individuals in other communities also supplied with lunch by the same caterer.

Stools were inoculated on Shigella-Salmonella (SS), MacConkey, thiosulphate citrate bile-salt sucrose (TCBS) and blood agar plates, and into selenite F and Rappaport broths. After incubation overnight, the selenite and Rappaport broths were subcultured on SS agar. In addition, enrichment culture in phosphate buffer

solution at pH 7·6 (Paterson & Cook, 1963) for 10 days or more at 8° C. was also used for most of the specimens.

At first, all the plates were incubated overnight at 37° C., but later, with the exception of blood and TCBS agar plates, they were all incubated for 48 hours at 30° C. and examined for the usual enteropathogens. Blood agar was incubated anaerobically at 37° C. for *Clostridium perfringens*.

Blood

Samples were collected from patients in the acute stage of illness and again after 14–20 days. Blood samples were also obtained from persons who were not ill. The sera were examined by an agglutinin titration procedure similar to that used with Widal tests.

Water

Samples of water used by the communities were investigated for total bacterial count, enumeration of coliform organisms and culture for enteropathogens several times during the period of the epidemic. For pathogens, 10 l. samples of water were filtered through Milipore membranes (0·45 μm) and the filters then placed in nutrient broth. After incubation for 48 hr. at 30° C., the broth was subcultured on SS and MacConkey agar.

Animals

Stool or cloacal swabs from various pet animals in the community, as well as autopsy material from rats caught in the vicinity were investigated for the presence of *Y. enterocolitica*.

Identification and characterization of the strains isolated were carried out with accepted media and methods used in enteric bacteriology (Edwards & Ewing, 1962).

RESULTS

The outbreaks

Outbreak A

Between 31 January and 26 February 1972, 182 children and one teacher among 390 persons at a primary school (A) and 6 of 51 children in the kindergarten associated with this school suffered from an illness resembling bacterial food poisoning. The majority of the 189 cases occurred during the first 10 days of the outbreak, but some continued to occur sporadically until 26 February (Table 1). With one possible exception, no secondary cases occurred in the families of the children affected. The exception was a junior high school boy whose brother was involved in the outbreak; they both suffered similar clinical symptoms at the same time.

The symptoms included abdominal pain (85·7 %), fever (75·5 %), diarrhoea (60·1 %), nausea (23·5 %), and vomiting (4·0 %) as shown in Table 2. The body temperature ranged from 38° C. to 39·5° C. in most of the cases. Some patients, misdiagnosed as appendicitis, were treated by surgical operation. About half the cases with diarrhoea had only a few loose stools. There were no deaths and most of the patients recovered within one or two days.

Table 1. *Daily incidence of cases in two outbreaks of* Y. enterocolitica *infection*

Outbreak A		Outbreak B	
Date	Number of cases	Date	Number of cases
Jan. 31	4	July 11	79
Feb. 1	21	12	254
2	44	13	134
3	34	14	55
4	29	15	9
5	12	16	5
6	6	17	1
7	5	18	3
8	3	19	0
9	12	20	3
10	4	21	0
11	0	22	1
12	2		
13	0		
14	3		
15	1		
16	2		
17	1		
18	0		
19	0		
20	2		
21	1		
22	2		
23	0		
24	0		
25	0		
26	1		
Total no. of cases	189		544

Outbreak B

Between 11 and 22 July 1972, another explosive outbreak occurred among children in a primary school in another district, 15 km. from school A involved in the first epidemic. Of 993 children and 49 adults at risk, 544 (52·6 %) pupils were ill. The majority of cases occurred within the first 4 days, but sporadic cases continued to occur until 22 July (Table 1). No secondary cases occurred in any family contacts.

The main clinical symptoms were abdominal pain (63·9 %), fever (49·6 %), diarrhoea (32·4 %), nausea (24·8 %) and vomiting (10·6 %). A temperature of 39° C. or higher occurred in 30 % of 270 patients with fever. In the majority of cases with diarrhoea, watery stools were observed. Most patients recovered within 12–18 hr.; there were no deaths (Table 2).

Table 2. *Clinical features*

Main symptoms	Number of cases	
	Outbreak A*	Outbreak B†
Abdominal pain	84 (85·7 %)	348 (63·9 %)
Fever	74 (75·5 %)	270 (49·6 %)
Diarrhoea	59 (60·1 %)	176 (32·4 %)
Nausea	23 (23·5 %)	135 (24·8 %)
Vomiting	4 (4·0 %)	58 (10·6 %)

* Interview on 98 cases.
† Interview on 544 cases.

Table 3. *Isolation of* Yersinia enterocolitica *from stool specimens in outbreak A*

Source	Number of specimens tested	Number of specimens positive for *Y. enterocolitica*
School A		
Acute cases	113	48 (42·4 %)
Convalescent cases	175	36 (20·5 %)
Symptomless cases	234	9 (3·8 %)
Kindergarten	104	1*
Food catering institution	10	0
Family contacts of cases	122	0

* Symptomless case.

Bacteriology

Stool specimens

Outbreak A. When outbreak A was first investigated, no causative agent was isolated from stools because *Y. enterocolitica* was not considered and most plates were discarded after overnight incubation at 37° C. However, on examination of further stool cultures, *Shigella*-like colonies, later identified as *Y. enterocolitica*, were observed on SS agar plates kept for 24 hr. at room temperature after overnight incubation at 37° C. Accordingly, some of the earlier specimens, which had been kept at −20° C., were re-examined for this organism, and incubation for 48 hr. at 30° C. was adopted for subsequent bacteriological investigations.

In outbreak A, a total 113 stool specimens from acute cases were available for specific investigation and, as shown in Table 3, *Y. enterocolitica* was found in 42·4 % of these specimens. Other pathogens, including *Salmonella*, *Shigella*, enteropathogenic *Escherichia coli*, and *Clostridium perfringens*, were not found. On 11 March, 40 days after the outbreak started, stools were obtained from 175 persons who had been ill, and the same organism was isolated from 20·5 % of the specimens.

Of 234 specimens from symptomless persons in school A, 3·8 % were positive for the organism. In contrast, it was isolated from only one of 114 specimens from persons in institutions other than school A, including the catering establishment. The single positive specimen was from a symptomless child who had no connexion

Table 4. *Isolation of* Yersinia enterocolitica *from stool specimens in outbreak B*

Source	Number of specimens tested	Number of specimens positive for *Y. enterocolitica*
Acute cases	117	88 (75·1 %)
Convalescent cases	544	5 (0·9 %)
Food catering staff	5	0
Family contacts of cases	212	0

with school A. The organism was not isolated from 122 healthy individuals from the families of the cases.

Outbreak B. In outbreak B, a total of 117 stool specimens from cases were available on 15 July, and the same organism as that in outbreak A was isolated from 75 % of them (Table 4). On 5 September, 56 days after the first cases occurred, 544 specimens from convalescent patients were obtained and *Y. enterocolitica* was isolated from 0·9 % of them. It was not isolated from any specimens from the families of cases.

Isolation and identification. On SS agar, the organisms isolated in both outbreaks formed smooth colourless colonies resembling those of *Shigella* after 48 hr. incubation at 30° C.; they were only minute in size after overnight incubation at 37° C. Although the organism grew on ordinary and less inhibitory media, SS agar was the most convenient for isolation because it strongly inhibited other faecal organisms. Although selenite and Rappaport broths were both recommended for enrichment culture of *Y. enterocolitica* by Niléhn & Sjöström (1967*a*) and van Noyen & Vandepitte (1968), they were not as good as direct culture of stool specimens on SS agar in the present investigation. However, inoculation of the stools in phosphate buffer solution (pH 7·6) which was then kept for 10 days or more at 8° C. before subculture to SS agar, yielded more isolations than direct culture on SS agar.

The organisms isolated were Gram-negative, facultatively anaerobic, fermentative rods. All the strains were similar in morphology and gave the same cultural, biochemical, and serological results (Table 5) which confirmed their identity as *Y. enterocolitica* of O group 3 and biotype 4.

Water samples

Well-water, pumped up into large tanks and then chlorinated, was used in school A. Samples were tested repeatedly during the outbreak, but the concentration of residual chlorine in the water was satisfactory and no coliform organisms were detected. Indeed, only occasional viable organisms per ml. were found in the water samples examined.

In school B, chlorinated water from the city mains was supplied. This was also used in a swimming pool in the school grounds. The tap water and swimming pool water were both investigated early in the outbreak, but chlorine levels were again satisfactory and no coliform organisms or other Gram-negative rods were detected.

Table 5. *Characteristics of strains isolated*

Test	Reaction	Test	Reaction
Gram stain	–	Acid only from:	
Oxidase	–	Arabinose	+
Motility, 37° C	–	Celliobiose	+
Motility, 25° C	+	Lactose	–
Nitrate reduction	+	Maltose	+
Indole	–	Mannose	+
Voges–Proskauer, 37° C	–	Melezitose	–
Voges–Proskauer, 25° C	+	Melibiose	–
Ammonium glucose	+	Raffinose	–
Ammonium citrate	–	Rhamnose	–
Ammonium acetate	+	Sorbose	+
Malonate utilization	–	Sucrose	+
Urease	+	Trehalose	+
Hydrogen sulphide (TSI)	–	Xylose	+
Lysine decarboxylase	–	Adonitol	–
Arginine dihydrolase	–	Dulcitol	–
Ornithine decarboxylase	+	Erythritol	–
Phenylalanine deaminase	–	Glycerol	+
Gelatinase	–	Mannitol	+
Haemolysis	–	Sorbitol	+
Lipase, corn-oil	–	Salicin	–
Kauffmann–Petersen, Citrate	–	Aesculin	–
Kauffmann–Petersen, D-Tartrate	–	Inositol	+
Kauffmann–Petersen, Mucate	–	α-Methylglucoside	+
O–F medium, glucose (sealed)	+	β-Methylglucoside	+
Gas from glucose	–	β-Galactosidase	+

Animals

In outbreak A, faecal specimens from pet animals in the school including three chickens, four pheasants, three pigeons and one rabbit, as well as autopsy material from 10 rats caught near the school were examined for *Y. enterocolitica* with negative results.

Serological survey

Sera from patients and from unaffected persons associated with both outbreaks were examined. Agglutinin titres ranged from 1/160 to 1/1280 after 3 weeks in the majority of convalescent cases, compared with 1/20 or less in the acute stage of illness. Agglutinin titres rarely exceeded 1/20 in sera from persons who were not ill.

DISCUSSION

As it was isolated from the faeces from most of the cases and since no other enteropathogenic organisms were recognized, *Y. enterocolitica* was almost certainly the causative organism in the two outbreaks described. Significantly raised agglutinin titres against this organism in convalescent sera further confirmed this view. Although sporadic cases of human infection with *Y. enterocolitica* have been reported (Carlsson, Ryd & Sternby, 1964; Winblad, Niléhn & Sternby, 1966; Winblad, Niléhn & Jonsson, 1966; Niléhn & Sjöström, 1967*a*, *b*; Winblad, 1968; Niléhn *et al.* 1968; Ahvonen, Sievers & Aho, 1969; Braunstein, Tucker & Gibson, 1971), community outbreaks have not previously been described.

The isolation of *Y. enterocolitica* from numerous animal sources including hares, chinchillas, pigs, dogs, cattle and the bush-baby (Dickinson & Mocquot, 1961; Becht, 1962; Akkermans & Terpstra, 1963; Daniëls & Goudzwaard, 1963; Daniëls, 1963; Knapp & Thal, 1963; Struve, 1963; Mollaret, Chevalier & Deplanche, 1964; Mollaret & Lucas, 1965; Siegmann, 1965; Mair, White, Schubert & Harbourne, 1970) suggests that human infection with *Y. enterocolitica* may be food-borne. Rabson & Koornhof (1972), Esseveld & Goudzwaard (1972) and Rakovský, Pauckova & Aldova (1972) considered that pigs were the main source of human infection. On the other hand, Szita, Káli & Rédey (1972) thought that spread of infection from man to man was more likely because there was no indication of transmission from animal sources in their study. It is difficult, however, to reconcile this view with their statement that an oral dose of $3{\cdot}5 \times 10^9$ organisms was needed to cause illness in human volunteers, as it seems unlikely that such large numbers of *Y. enterocolitica* would be transmitted in this way.

In the present incidents, cases continued to occur for about one month in outbreak A and for 11 days in outbreak B. This picture is more like that seen in outbreaks of *Shigella* infection than of food poisoning, and does suggest that infection from man to man can occur. However, all cases in the present outbreaks were restricted to pupils and one teacher, and spread of infection to family contacts was not observed. Although no conclusions can be drawn, these findings suggest that the outbreaks described were probably food- or water-borne.

More recently, in a further outbreak of *Y. enterocolitica* infection, 198 cases occurred in a junior high school in a different locality 200 km. from Shizuoka (Zen-Yoji *et al.* 1973). In addition, an outbreak of infection at a primary school 400 km. from Shizuoka was thought to be due to *Y. enterocolitica* (Sakazaki *et al.* unpublished). In this instance, high agglutinin titres to O antigen 3 were demonstrated in convalescent sera taken 2 months after the outbreak, although bacteriological examination for *Y. enterocolitica* was not performed during this outbreak. In both these incidents, the source and mode of infection were again unknown.

The outbreaks described in the present paper were first thought to be food-poisoning and *Y. enterocolitica* was not even considered. It is thus possible that other outbreaks of 'food-poisoning' of unknown aetiology may be caused by this organism. More attention should therefore be paid to *Y. enterocolitica* infection, not only in sporadic cases but also in community outbreaks of infection.

Our thanks are expressed to the staff of Shizuoka Department of Public Health, Japan, for obtaining specimens and clinical data; to Dr G. Wauters, Institut Rega, Louvain, for assistance in identifying the organism; and to Dr G. I. Barrow, Public Health Laboratory, Truro, Cornwall, for his help with the manuscript.

REFERENCES

Ahvonen, P., Sievers, K. & Aho, K. (1969). Arthritis associated with *Yersinia enterocolitica* infection. *Acta rheumatologica scandinavica* **15**, 232–53.

Akkermans, J. P. W. M. & Terpstra, J. I. (1963). Pseudotuberculose bij chinchilla's veroorzaakt door een bijondere species. *Tijdschrift voor Diergeneeskunde* **88**, 91–5.

BECHT, H. (1962). Untersuchungen über die Pseudotuberkulose beim Chinchilla. *Deutsche tierärztliche Wochenschrift* **69**, 626–7.

BRAUNSTEIN, H., TUCKER, E. B. & GIBSON, B. C. (1971). Mesenteric lymphadenitis due to *Yersinia enterocolitica*: report of a case. *American Journal of Clinical Pathology* **55**, 506–10.

CARLSSON, M. G., RYD, H. & STERNBY, N. H. (1964). A case of human infection with *Pasteurella pseudotuberculosis*. X. *Acta pathologica et microbiologica scandinavica* **62**, 128–32.

DANIËLS, J. J. H. M. (1963). Untersuchungen an als *Pasturella pseudotuberculosis* diagnostizierten Stämmen von Chinchillas. *Zentralblatt für Veterinärmedizin* **10**, B, 413–17.

DANIËLS, J. J. H. M. & GOUDZWAARD, C. (1963). Enkele stammen van een op *Pasteurella pseudotuberculosis* gelijkend niet geidentificeerd species, geisoleerd bij knaagdieren. *Tijdschrift voor Diergeneeskunde* **88**, 96–102.

DICKINSON, A. B. & MOCQUOT, G. (1961). Studies on the bacterial flora of the alimentary tract of pigs. I. *Enterobacteriaceae* and other Gram-negative bacteria. *Journal of Applied Bacteriology* **24**, 252–84.

EDWARDS, P. R. & EWING, W. H. (1962). *Identification of Enterobacteriaceae*. Minneapolis, Burgess Publishing Company.

ESSEVELD, H. & GOUDZWAARD, C. (1972). Serological diagnostic in proven human infections with *Y. enterocolitica* type 9. *Abstracts of International Symposium on* Yersinia, *Pasteurellae and* Francisella, *Malmö*.

KNAPP, W. & THAL, E. (1963). Untersuchungen über die kulturell-bio-chemischen, serologischen, tierexperimentellen und immunologischen Eigenschaften einer vorläufig 'Pasteurella X' benannten Bakterienart. *Zentralblat für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene*, Abt. 1, orig. **190**, 472–84.

MAIR, N. S., WHITE, G. D., SCHUBERT, F. K. & HARBOURNE, J. F. (1970). *Yersinia enterocolitica* infection in the bush-baby (Calago). *Veterinary Record*, **86**, 69–71.

MOLLARET, H. H., CHEVALIER, A. & DEPLANCHE, M. C. (1964). Contribution à l'étude d'un nouveau groupe de germes proches du bacille de Malassez et Vignal. 1. Caractères culturaux et biochimiques. *Annales de l'Institut Pasteur* **107**, 121–7.

MOLLARET, H. H. & LUCAS, A. (1965). Sur les particularités biochimiques des souches de *Yersinia enterocolitica* isolées chez les lièvres. *Annales de l'Institut Pasteur* **108**, 121–5.

NILÉHN, B. & SJÖSTRÖM, B. (1967*a*). Occurrence of *Yersinia enterocolitica* in patients admitted to hospital because of suspected acute appendicitis. *Acta pathologica et microbiologica scandinavica*, Supp. **187**, 77–8.

NILÉHN, B. & SJÖSTRÖM, B. (1967*b*). Studies on *Yersinia enterocolitica*: occurrence in various groups of acute abdominal disease. *Acta pathologica et microbiologica scandinavica* **71**, 612–28.

NILÉHN, B., SJÖSTRÖM, B., DAMGAARD, K. & KINDMARK, C. (1968). *Yersinia enterocolitica* in patients with symptoms of infectious diseases. *Acta pathologica et microbiologica scandinavica* **74**, 101–13.

PATERSON, J. S. & COOK, R. (1963). A method for the recovery of *Pasteurella pseudotuberculosis* from faeces. *Journal of Pathology and Bacteriology* **85**, 241–2.

RABSON, A. R. & KOORNHOF, H. J. (1972). *Yersinia enterocolitica* infections in South Africa. *Abstracts of International Symposium on* Yersinia, *Pasteurellae and* Francisella, *Malmö*.

RAKOVSKÝ, J., PAUCKOVA, V. & ALDOVA, E. (1972). *Yersinia enterocolitica* infections in man in Czechoslovakia. *Abstracts of International Symposium on* Yersinia, *Pasteurellae and* Francisella, *Malmö*.

SIEGMANN, O. (1965). Zur Epizoologie der Pseudotuberkulose beim Chinchilla. *Kleintier-Praxis* **9**, 17–19.

STRUVE, M. (1963). Untersuchungen mit Bakterienstämmen einer vorläufig als *Pasteurella* X bezeichneten Bakterienart. Inaugural Dissertation, Tübingen.

SZITA, J., KÁLI, M. & RÉDEY, B. (1972). Incidence of *Yersinia enterocolitica* infections in Hungary. Observations on the laboratory diagnosis of yersiniosis. *Abstracts of International Symposium on* Yersinia, *Pasteurellae and* Francisella, *Malmö*.

VAN NOYEN, R. & VANDEPITTE, J. (1968). L'isolement de *Yersinia enterocolitica* par une technique usuelle de coproculture. *Annales de l'Institut Pasteur* **114**, 463–7.

WINBLAD, S. (1968). The epidemiology of human infections with '*Yersinia pseudotuberculosis*' and '*Yersinia enterocolitica*' in Scandinavia. *Symposia Series in Immunological Standardization* **9**, pp. 133–6. Basel & New York, Karger.

WINBLAD, S., NILÉHN, B. & JONSSON, M. (1966). Two further cases, bacteriologically verified, of human infection with '*Pasteurella* X' (syn. *Yersinia enterocolitica*). *Acta pathologica et microbiologica scandinavica* **67**, 537–41.

WINBLAD, S., NILÉHN, B. & STERNBY, N. H. (1966). *Yersinia enterocolitica* (*Pasteurella* X) in human enteric infections. *British Medical Journal* ii, 1363–6.

ZEN-YOJI, H. & MARUYAMA, T. (1972). The first successful isolations and identification of *Yersinia enterocolitica* from human cases in Japan. *Japanese Journal of Microbiology* **16**, 493–500.

ZEN-YOJI, H., MARUYAMA, T., SAKAI, S., KIMURA, S., MIZUMO, T. & MOMOSE, T. (1973). An outbreak of enteritis due to *Yersinia enterocolitica* at a junior high school. *Japanese Journal of Microbiology*, in the Press.

J. Hyg. Camb., (1973), **71**, 725
With 1 plate
Printed in Great Britain

Isolation, ultrastructure and antigenicity of *Mycoplasma gallisepticum* membranes

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(*Received* 9 *March* 1973)

SUMMARY

The cell membrane of *Mycoplasma gallisepticum* was isolated by lysing the cells with digitonin. Chemical and density-gradient analyses and electron microscopy showed the isolated membranes to be relatively free of cytoplasmic contaminants. The density of the membranes exceeded that of other mycoplasma membranes, indicating a higher protein content. Small vesicular extensions seen in the sectioned membranes were interpreted as empty blebs.

The isolated membranes, but not the cytoplasmic fraction, elicited in chickens the production of growth-inhibiting, agglutinating and haemagglutination-inhibition antibodies to *M. gallisepticum* in titres resembling those obtained by injection of whole cells. The peak of the serological response varied with the serological test employed. The rapid slide-agglutination test became positive as early as 3 days after the first injection of only 50 μg. of membrane protein. The haemagglutination-inhibition antibody titre reached its peak at about 10 days after the first injection, while that of the growth-inhibiting antibodies was reached only at about 25 days. The addition of adjuvant to the membrane antigen did not improve the production of the growth-inhibiting antibodies in chickens, but it produced some improvement in rabbits. Our results support the thesis that the chief immunogens of *M. gallisepticum* reside in the cell membrane of this organism.

INTRODUCTION

As is well known, mycoplasmas differ from most other bacteria in that they lack a rigid cell wall, a characteristic which makes them particularly suitable for studies of the cell membrane (Razin, 1973). In some strains, such as *Acholeplasma laidlawii*, isolation of pure cell membranes is achieved by the simple procedure of osmotic lysis, and examination of the antigenic and biochemical properties of the membrane has advanced accordingly (Ne'eman, Kahane, Kovartovsky & Razin, 1972). In other strains, including some of the important pathogenic mycoplasmas, the cells are more refractory to osmotic lysis, and it has proved more difficult to obtain pure cell membranes in sufficient quantity for critical analysis of their antigens. Tech-

niques for preparation of membranes have included alternate freezing and thawing (Williams & Taylor-Robinson, 1967), osmotic lysis of glycerol-loaded cells (Kahane & Razin, 1969), ultrasonic treatment (Argaman & Razin, 1969), and gas cavitation (Hollingdale & Lemcke, 1969), but these lead either to fragmentation of the membranes or to lysis of only part of the cells (Razin, Kahane & Kovartovsky, 1972). In a recent report Rottem & Razin (1972) demonstrated the effectiveness of digitonin for the isolation of pure cell membranes from *Mycoplasma hominis*. The cells remain sensitive to digitonin treatment throughout the growth cycle. In addition, digitonin is effective in the presence of Mg^{2+} or other ions necessary to preserve membrane structure and function. We have examined the sensitivity of *Mycoplasma gallisepticum* to lysis by digitonin and have employed this reagent for the isolation of large quantities of pure membranes. The biochemical properties and the appearance in the electron microscope of these membranes are reported.

Previous communications have indicated that in *M. gallisepticum*, as in the other mycoplasmas studied, the cell membrane is the site of the chief cell antigens (Williams & Taylor-Robinson, 1967; Kahane & Razin, 1969). However, it was then difficult to obtain complete separation of the membrane from the non-membrane antigens. The ability to isolate pure membranes by digitonin has opened the way for a more critical analysis of the localization of antigens in this pathogenic mycoplasma, and for the fractionation of the membrane antigens. In addition, it was considered important to study the immunogenicity of the mycoplasma and its isolated membrane in the natural host – in this case, the chicken. Use of the chicken would permit an examination of the protective effect of the immune response to mycoplasma antigens, an essential step in the development of a vaccine to this or other pathogenic mycoplasmas. Thus, in addition to the details of the isolation of membranes from *M. gallisepticum* by digitonin and the biochemical analysis of these membranes, this report presents various aspects of the immunogenicity of mycoplasma membrane antigens in chickens.

MATERIALS AND METHODS

Organism and growth conditions

Mycoplasma gallisepticum strain A5969 was obtained from Dr M. E. Tourtellotte (The University of Connecticut, Storrs, U.S.A.). Identification of the strain as *M. gallisepticum* was verified by growth inhibition tests (Clyde, 1964) with a standard *M. gallisepticum* anti-serum obtained from the Central Veterinary Laboratory, Weybridge, Surrey. The organisms were grown in 3 l. volumes of a modified Edward medium (Razin, 1963) containing either 10 % (v/v) horse serum or 2 % (v/v) of Difco PPLO serum fraction. For labelling membrane lipids 50 μCi of (9, 10-^{3}H)-oleic acid (The Radiochemical Centre, Amersham, England) were added to each litre of the growth medium. Cultures were usually harvested after 20 hr. of static incubation at 37° C. when turbidity reached 0·2–0·3 O.D. units at 590 nm. The organisms were sedimented by centrifugation at 9000 *g* for 30 min., resuspended and washed once in 0·25 M-NaCl.

Assessment of sensitivity to lysis by digitonin

The sensitivity of the organisms to lysis by digitonin was tested as described before (Rottem & Razin, 1972) in a series of test tubes containing different concentrations of digitonin in 0·25 M-NaCl. Lysis was assessed by measuring the change in turbidity of the treated cell suspensions at 500 nm after 15 min. incubation at 37° C. Results were expressed as percentage lysis calculated according to the formula

$$\% \text{ lysis} = \frac{\text{O.D. of untreated suspension} - \text{O.D. of treated suspension}}{\text{O.D. of untreated suspension} - \text{O.D. of completely lysed suspension}} \times 100,$$

where 'untreated suspension' represents a control suspension without digitonin and 'completely lysed suspension' one treated with 100 μg./ml. or more digitonin until there was no further decrease in turbidity upon the addition of more digitonin. The remaining turbidity is presumably due to membrane and other insoluble material.

Isolation of cell membranes

The organisms harvested from 3 l. of medium at O.D.590 = 0·20–0·30 were washed once in 0·25 M-NaCl and resuspended in a small volume of the salt solution. The turbidity of this suspension was adapted so that a 1/30 dilution of it in 0·25 M-NaCl gave a reading of 0·5 O.D. unit at 500 nm. One volume of the concentrated cell suspension was added to 30 volumes of 0·25 M-NaCl containing 25 or 50 μg. digitonin/ml. The suspension was incubated in a 37° C. water bath for 15 min. The turbidity of the suspension at the end of the incubation period dropped to 0·05–0·09 O.D. units at 500 nm. The membranes were collected by centrifugation at 35,000 *g* for 30 min. The supernatant fluid was separated and used for the preparation of the cytoplasmic antigens as described in a forthcoming section. The viscous membrane pellet was resuspended in 60—80 ml. of 0·05 M-NaCl in 0·01 M phosphate buffer, pH 7·4, containing 5–10 μg. deoxyribonuclease per ml. (DN-100, Sigma) and 0·01 M-$MgCl_2$. After 30–60 min. incubation at room temperature with constant stirring viscosity disappeared and the membranes were collected by centrifugation at 35,000 *g* for 30 min. and washed six times alternately with deionized water and 0·05 M-NaCl in 0·01 M phosphate buffer, pH 7·4. The washed membranes were resuspended in 1/20 β-buffer (Razin, Morowitz & Terry, 1965) to a concentration of 3–4 mg. membrane protein/ml. and stored at −70° C. until used.

An alternative method of digesting the viscous DNA liberated during cell lysis is to add the deoxyribonuclease and Mg^{2+} to the digitonin solution, so that the enzyme can act during cell lysis. The only disadvantage of this method is that, owing to the large volume of the digitonin solution, considerable quantities of the expensive enzyme are necessary to maintain the level of 10 μg. enzyme/ml.

Analytical methods

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard. Membrane lipids were extracted with chloroform + methanol (2 + 1, by vol.) according to Folch, Lees & Sloane-Stanley (1957). Nucleic acids were extracted from the lipid-depleted residue and

determined as described before (Razin, Argaman & Avigan, 1963). Total carbohydrate was estimated by the method of Dubois *et al.* (1956) on the residue remaining after nucleic acid extraction. Radioactivity in membrane lipids was determined in a Packard Tri-Carb liquid scintillation spectrometer using a dioxane-toluene scintillation liquor (Kahane & Razin, 1969). Density-gradient centrifugation was performed as described before (Rottem, Stein & Razin, 1968) using 12 ml. of a linear sucrose gradient of 30 to 60%.

Preparation of antigens

M. gallisepticum cells washed at least four times in 0·25 M-NaCl and suspended in the same solution were used as whole cell antigen. The preparation of membrane antigens was as described above. The cytoplasmic antigens were prepared by freeze-drying of the supernatant fluid obtained after removal of the membranes of cells lysed by digitonin. The dried material was redissolved in physiological saline.

Antigens for immunization were prepared by dilution of the above preparations to the desired protein concentration in phosphate-buffered saline (PBS), pH 7·4. For use with adjuvant, equal volumes of antigen solution and Freund's complete or incomplete adjuvant (Difco) were mixed and treated in an M.S.E. ultrasonic disintegrator (60 W, 20 kc/sec.) for six 30 sec. periods in ice.

Immunization of rabbits

Rabbits were immunized according to the schedule of Kahane & Razin (1969). The rabbits were bled before immunization, at the time of the last injection and 1 week after that.

Immunization of chickens

The chickens used were a cross between White Rock and Cornish breeds from mycoplasma-free flocks. Periodical examination using the rapid slide-agglutination test showed the chickens to be serologically negative to *M. gallisepticum*. The chickens were 11–13 weeks old at the start of the immunization period. The immunization schedule is summarized in Table 1. As with the rabbits one half of the total antigen dose was given on the first day and the rest was distributed over the three subsequent weeks. The chickens were bled from the wing vein before immunization, at the time of each injection and at other time intervals as stated in the Results section.

Serological tests

Rapid slide agglutination test

Chicken and rabbit sera were tested for the presence of antibodies to *M. gallisepticum* by a commercial stained antigen prepared from *M. gallisepticum* strain S6 (De Zeeuw Laboratories, De Bilt, Netherlands). The tests were performed according to the manufacturer's instructions.

Tube agglutination

Agglutination of whole cells was tested according to Bailey *et al.* (1961), but the temperature of incubation was 37° C. instead of 52° C.

Table 1. *Schedule of immunization of chickens by* M. gallisepticum *antigens*

Day of injection	Antigen + adjuvant	Antigen alone	Volume injected (ml.) Each leg IM	Each leg SC	Each side of breast IM	Each side of breast SC	Each wing membrane SC
1	+	—	0·2	0·2	0·2	0·2	0·2
8	—	+	0·1	—	—	—	—
15	—	+	0·1	—	0·1	—	—
22	—	+	0·1	—	0·1	—	—

IM = intramuscular; SC = subcutaneous.

Growth inhibition

The technique of Clyde (1964) was used. The medium was a modified Edward medium (Razin, 1963) containing 10% (v/v) fresh horse serum and 1·5% (w/v) Difco agar. The filter paper disks were saturated with undiluted antiserum. Best results were usually obtained when the inoculum spread on the plate was taken from a 10^{-2} dilution of a logarithmic-phase culture of *M. gallisepticum*.

Metabolic inhibition

The test was carried out in a medium containing glucose as described by Taylor-Robinson, Purcell, Wong & Chanock (1966). In early experiments a 10^{-2} dilution of an actively growing culture was used as an inoculum; later, in an attempt to reduce variability, small samples of a young culture were kept at $-70°$ C. and used at 10^{-1} dilution for each performance of the test.

Haemagglutination inhibition

The antigen for this test was a concentrated suspension of washed *M. gallisepticum* cells prepared in 50% glycerol solution in PBS and stored in small samples at $-20°$ C. Haemagglutination inhibition was tested according to the constant antigen-decreasing antiserum method recommended in 'Methods for Examining Poultry Biologics and for Identifying and Quantifying Avian Pathogens' (published by the U.S.A. National Academy of Science, 1971, p. 229). The test was performed in lucite plates with a reaction volume of 1·0 ml. Dilutions of the antisera in the antigen suspension were allowed to stand for 30 min. at room temperature before the addition of red blood cells. The results were expressed in haemagglutination-inhibition units, which are equivalent to the highest dilution of serum producing complete inhibition of haemagglutination multiplied by the haemagglutination units of the antigen (Kuniyasu & Ando, 1966). A haemagglutination unit (HA) is defined as the highest dilution of the stock antigen which will completely agglutinate the test dose of erythrocytes under the standard conditions of the test. In our experiments 2–8 haemagglutination units were used.

Electron microscopy

For the preparation of thin sections, pellets of sedimented membranes were fixed in 4% glutaraldehyde in 0·1 M phosphate buffer (pH 7·4) for 4 hr. at 0° C., washed in the phosphate buffer, and postfixed in 2% OsO_4 in the same buffer for 16 hr. The material was dehydrated and embedded in Epon by the method of Luft (1961). Sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a Phillips EM-300 electron microscope.

RESULTS

Sensitivity of M. gallisepticum *to lysis by digitonin*

M. gallisepticum cells were found to be sensitive to lysis by digitonin. As with *M. hominis* (Rottem & Razin, 1972) the lytic effect of digitonin decreased with the increase of the amount of cells in suspension (Fig. 1). Cultures harvested at various times before their O.D.590 reached 0·3 showed about the same sensitivity to digitonin.

Ultrastructure and composition of M. gallisepticum *membranes*

Plate 1 shows thin sections prepared from the sediment obtained after lysis of *M. gallisepticum* cells with 25 μg. digitonin per ml. The sections demonstrate that the sedimented material consists of membranes having the characteristic trilaminar shape in section with very little evidence of cytoplasmic contaminants, such as ribosomes. Very few cells could be detected in the material examined. The gross chemical composition of the membrane preparation is compared with that of whole *M. gallisepticum* cells in Table 2. The membranes were highly enriched in lipid, but contained much lower amounts of nucleic acids than whole cells. Total carbohydrate content was very low in both cells and membranes. As could be expected the density of the membrane preparation was significantly lower than that of the cells. In a typical preparation about 65 mg. of membrane protein were obtained from 160 mg. of total cell protein.

Immunogenicity of isolated membranes and cytoplasmic fraction

Table 3 shows that all the chickens injected with membranes responded in production of growth-inhibiting, agglutination and haemagglutination-inhibiting antibodies to M. *gallisepticum* in titres resembling those obtained by injection of whole cells. Injection of the cytoplasmic fraction failed to elicit any significant antibody response as measured by the above-mentioned tests (Table 3). In general, pre-immunization sera were negative when tested by the tube agglutination and growth inhibition tests, and were negative or showed only low titres in the haem-agglutination-inhibition test.

The peak of the serological response in the positive responders depended on the serological test employed. Thus, the chickens which were immunized with whole cells or membranes were converted from negative to positive responders in the slide agglutination test as early as 3 days after the first injection. The birds

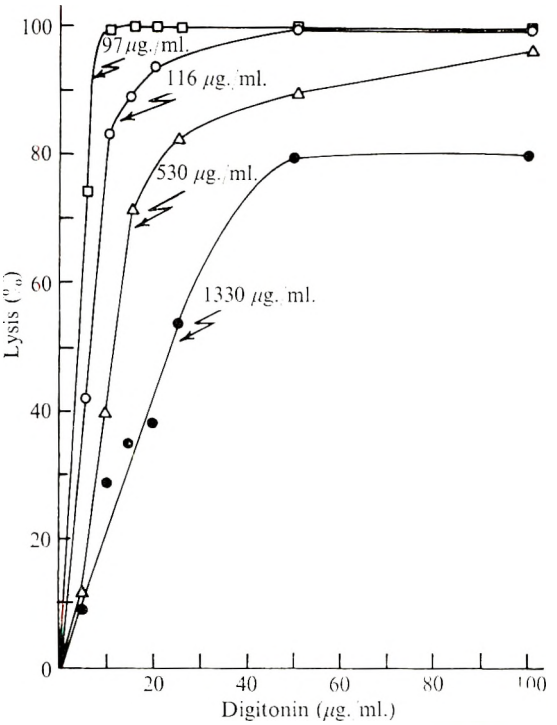


Fig. 1. Effect of the concentration of *M. gallisepticum* cells in suspension on their sensitivity to lysis by digitonin. The concentration of cell protein is given in μg. per ml. of suspension.

Table 2. *Chemical composition of* M. gallisepticum *cells and membranes isolated by digitonin lysis*

	Lipid*					
Preparation	μg./mg. protein	Counts/min. per mg. protein	DNA (μg./mg. protein)	RNA (μg./mg. protein)	Carbohydrate (μg./mg. protein)	Density (g./cm.^{3})
Whole cells	74	261,122	60	130	6	1·230
Membranes	198	700,017	10	10	2	1·199

* Membrane lipids were labelled during growth with [9,10-^{3}H]-oleic acid.

remained positive in this test for at least 6 weeks after the last injection. Those which received the cytoplasmic fraction remained negative throughout the immunization period. Fig. 2 shows that the appearance of haemagglutination-inhibition antibodies was also very fast, reaching a peak-titre at about 10 days after the first injection, while the growth-inhibiting antibodies reached the peak at about 25 days after the first injection.

The effect of adjuvant on the immune response of chickens and rabbits to *M. gallisepticum* membranes can be seen in Table 4. The data in the table indicate that the production of growth-inhibiting antibodies in chickens does not depend on the addition of adjuvant to the membrane antigen. With rabbits, on the other hand, Freund's complete adjuvant appears to improve the immune response. Table 4 also shows that the antisera prepared in rabbits inhibited growth of *M. gallisepticum* more effectively than the antisera prepared in chickens.

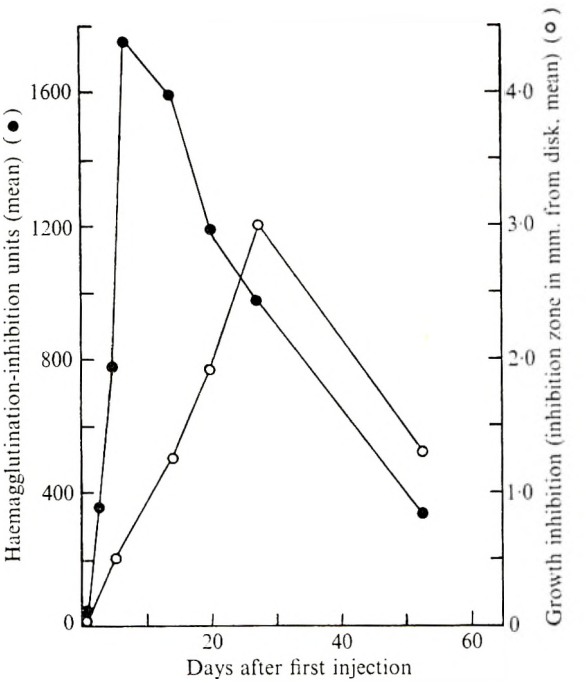


Fig. 2. The development and persistence of haemagglutination-inhibition and growth-inhibition antibodies to *M. gallisepticum* in nine chickens injected with either whole cells or membranes without adjuvant.

Table 3. *Serological response of chickens to whole cells and cell fractions of* M. gallisepticum

Expt.	Antigen*	No. of chickens	Growth inhibition (inhibition zone in mm. from disk, mean and range)	Tube agglutination titre (mean and range of reciprocal)	Haemagglutination inhibition units (mean and range)
1	Whole cells	5	2·1 (1·0–4·0)	33 (20–40)	ND
	Membranes	4	1·6 (1·0–2·0)	45 (10–80)	ND
	Cytoplasmic fraction	4	0	< 5	ND
2	Whole cells	5	ND	20 (10–40)	3710 (640–10240)
	Membranes	5	ND	13 (10–40)	1090 (320–2560)
	Cytoplasmic fraction	4	ND	< 5	160 (0–640)
	Uninoculated chickens	3	ND	< 5	< 10

* The total amount of antigen injected into each chicken contained in the first experiment 5 mg. protein with Freund's incomplete adjuvant and in the second experiment 1 mg. protein with no adjuvant. The data given in the table represent the maximal titres attained. ND = not done.

Table 5 shows the relation between the antigen dose and the immune response. The most striking effect of the decrease in antigen dose was a decrease in the proportion of the inoculated birds showing a positive response. That is, at 5 mg. membrane or whole cell protein all the inoculated birds showed a detectable response. At lower doses the proportion of positive responders in the growth

Table 4. *Effect of adjuvant on the immune response of chickens and rabbits to* M. gallisepticum *membranes as measured by growth-inhibiting antibodies*

Animal	No. of animals	Adjuvant	Growth inhibition (inhibition zone in mm. from disk, mean and range)
Chickens*	4	Freund's complete	1·6 (1·0–2·0)
	5	Freund's incomplete	1·8 (1·0–3·5)
	4	No adjuvant	1·9 (1·5–2·0)
Rabbits†	4	Freund's complete	5·2 (3·7–6·7)
	3	Freund's incomplete	3·3 (3·0–3·9)
	2	No adjuvant	3·2 (2·6–3·8)

* Chickens were immunized with 5 mg. membrane protein. Maximal titres are presented.
† The titre at 5 weeks after the initial injection.

Table 5. *Relationship between antigen dose and the immune response in chickens*

		Growth inhibition*		Tube agglutination†	
Antigen	Dose (mg. protein)	Proportion of positive responders	Inhibition zone in mm. from disk in positive responders, mean and range	Proportion of positive responders	Reciprocal of titre in positive responders (mean and range)
Membranes	5	4/4	2·0 (1·0–4·0)	4/4	22 (10–40)
	1	2/5	1·5 (1·0–3·0)	5/5	17 (5–40)
	0·1	2/5	2·0 (1·0–3·0)	3/5	30 (10–40)
Whole cells	5	4/4	1·8 (1·0–3·0)	4/4	30 (20–40)
	1	3/5	1·4 (1·0–2·0)	5/5	60 (10–160)
	0·1	3/5	1·8 (0·5–3·0)	4/5	12 (10–20)

* Tested in sera obtained 27 days after the first injection.
† Tested in sera obtained 7 days after the first injection.

inhibition and tube agglutination tests decreased. However, all the birds, even those receiving the lowest dose (0·1 mg. protein) were converted from negative to positive when the rapid slide-agglutination test was employed. Moreover, the slide-agglutination test became positive even after the injection of only 50 μg. of membrane protein. Table 5 also shows that the positive responders to all doses of antigen did not differ significantly in the agglutination and growth inhibition titres.

DISCUSSION

The details of a simple reliable method for the isolation of large quantities of pure membranes from *Mycoplasma gallisepticum* are presented. To achieve lysis the method uses low concentrations of digitonin, a reagent known to form a specific complex with cholesterol and to cause cell lysis of the cholesterol-requiring mycoplasmas (Smith & Rothblat, 1960; Rottem & Razin, 1972). As emphasized by

Rottem & Razin (1972) complete lysis of the mycoplasma cells required a critical ratio between digitonin and cell mass – a point which might be overlooked in a large-scale preparation. Membranes can be readily prepared from cells at the end of the logarithmic phase of growth, the point of greatest cell yield. Further incubation, accompanied by a precipitous drop in the pH of the medium, is considered undesirable because of the possibility of adsorption of medium proteins to the cell membrane (Bradbury & Jordan, 1972; Rottem, Hasin & Razin, 1973) leading to changes in its antigenic structure.

Chemically and ultrastructurally the isolated *M. gallisepticum* membranes resemble the membranes of other mycoplasmas examined so far (Razin, 1973). The isolated membrane is composed almost entirely of protein and lipid. However, as indicated by their relatively high density (Table 2), the *M. gallisepticum* membranes appear to contain a higher percentage of protein than membranes of other mycoplasmas. This corroborates the finding of Rottem *et al.* (1968) that *M. gallisepticum* membranes, isolated by osmotic lysis of glycerol-loaded cells, consisted of about 79% protein and 19% lipid, as against 59% protein and 36% lipid in *Acholeplasma laidlawii* membranes. The idea put forward by Rottem *et al.* (1968) that the high protein content of *M. gallisepticum* membranes results from the presence of the bleb, a unique structure of this mycoplasma (Manniloff, Morowitz & Barrnett, 1965) appears to find support in our study. Thin sections of the isolated *M. gallisepticum* membranes show small vesicular extensions which may represent empty blebs (Plate 1*b*). Similar structures have not been detected so far in sections of isolated membranes from other mycoplasmas.

Our results demonstrate that the membranes isolated with digitonin are potent antigens. Thus immunization of chickens with membranes leads to the production of antibodies causing growth inhibition, agglutination of cells and haemagglutination inhibition. These results bear out the conclusions of earlier investigations with rabbits that the membrane is the site of the major immunogens of the cell (Williams & Taylor-Robinson, 1967; Argaman & Razin, 1969; Kahane & Razin, 1969; Razin *et al.* 1972). In contrast to the results with rabbits, only very low metabolism-inhibiting activity could be demonstrated in the immunized chicken sera. This observation, also noted by other investigators (Jordan & Kulasegaram, 1968; Taylor-Robinson & Berry, 1969), probably occurs because of the presence of factors interfering with the fixation of complement by sera from artificially immunized birds (Rice, 1947). We obtained demonstrable metabolism-inhibiting activity with immune chicken sera, but the titres were very low (only up to 1/32). Under the same experimental conditions rabbit sera produced in response to the same membrane antigen exhibited very high titres of metabolism-inhibiting activity. Attempts to increase this activity in chicken sera by the addition of exogenous complement were not successful, and the results of the test were not included in the present study.

The antibodies measured by the slide-agglutination and the haemagglutination-inhibition tests appeared and reached their peak much earlier in our experiments than the antibodies causing growth inhibition of *M. gallisepticum*. These results are in accordance with previous reports. Thus, Jordan & Kulasegaram (1968)

found that chickens and turkeys infected with *M. gallisepticum* responded by the rapid production of agglutinating antibodies measured by the slide and tube-agglutination tests and by the haemagglutination-inhibition test. Kleven & Pomeroy (1971) found that the first detectable serological response of turkeys infected with *M. meleagridis* was a positive reaction in the slide-agglutination test (as early as 4 days after infection). The peak titre of the growth-inhibiting antibodies was reached much later in the experiments of Ogra & Bohl (1970) working also with turkeys infected with *M. meleagridis*. The finding of Roberts (1969), Kuniyasu (1969) and of Kleven & Pomeroy (1971) that the slide-agglutination test is associated with the early-appearing high molecular weight immunoglobulins (IgM) may provide the explanation for the rapid conversion of immunized chickens and turkeys from negative to positive in this test.

It is clear from the experiments presented in this work that chickens respond to the injection of membranes of *M. gallisepticum* by the production of specific antibodies against the mycoplasma cell. By the use of non-infectious antigens it is possible to distinguish the phenomenon of immunization from that of infection. It is hoped in the continuation of this work to relate the presence of antibodies of the various types to protection against infection by *M. gallisepticum*. It is also planned to examine the response of chickens to purified membrane fractions. Methods for the isolation of membrane fractions highly enriched in immunogenic activity have already been developed for *A. laidlawii* and *M. hominis* (Ne'eman *et al.* 1972; Razin *et al.* 1972; Hollingdale & Lemcke, 1969; 1972). If a similar fractionation of membrane antigens can be made in *M. gallisepticum* it may be possible to separate antigens that specifically induce protective antibodies and to eliminate antigens involved in hyperimmune effects. Furthermore, since chickens are the natural host of *M. gallisepticum* it should be possible to evaluate the protective effect of the membranes and specific antigens contained in it.

We thank Z. Ne'eman for carrying out the electron microscopy and M. Wormser for technical assistance in determining the chemical composition of the membranes. This work was supported by a grant from the Israeli Poultry Breeders Association and by grant FG-Is-286 from the U.S. Department of Agriculture.

REFERENCES

Argaman, M. & Razin, S. (1969). Antigenic properties of mycoplasma organisms and membranes. *Journal of General Microbiology* **55**, 45.

Bailey, J. S., Clark, H. W., Felts, W. R., Fowler, R. C. & Brown, T. McP. (1961). Antigenic properties of pleuropneumonia-like organisms from tissue cell cultures and the human genital area. *Journal of Bacteriology* **82**, 542.

Bradbury, J. M. & Jordan, F. T. W. (1972). Studies on the adsorption of certain medium proteins to *Mycoplasma gallisepticum* and their influence on agglutination and haemagglutination reactions. *Journal of Hygiene* **70**, 267.

Clyde, W. A. Jr. (1964). Mycoplasma species identification based upon growth inhibition by specific antisera. *Journal of Immunology* **92**, 958.

Dubois, M., Gilles, K., Hamilton, K., Rebes, P. & Smith, E. (1956). Determination of carbohydrates by phenol. *Analytical Chemistry* **28**, 350.

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* **226**, 497.

HOLLINGDALE, M. R. & LEMCKE, R. M. (1969). The antigens of *Mycoplasma hominis*. *Journal of Hygiene* **67**, 585.

HOLLINGDALE, M. R. & LEMCKE, R. M. (1972). Membrane antigens of *Mycoplasma hominis*. *Journal of Hygiene* **70**, 85.

JORDAN, F. T. W. & KULASEGARAM, P. (1968). Serological tests for the detection of antibodies to *Mycoplasma gallisepticum* in chickens and turkeys. *Journal of Hygiene* **66**, 249.

KAHANE, I. & RAZIN, S. (1969). Immunological analysis of mycoplasma membranes. *Journal of Bacteriology* **100**, 187.

KLEVEN, S. H. & POMEROY, B. S. (1971). Characterization of the antibody response of turkeys to *Mycoplasma meleagridis*. *Avian Diseases* **15**, 291.

KUNIYASU, C. (1969). Nature of antibody response of chickens to *Mycoplasma gallisepticum*. *National Institute of Animal Health Quarterly, Tokyo* **9**, 119.

KUNIYASU, C. & ANDO, K. (1966). Studies on the hemagglutination test for *Mycoplasma gallisepticum* infection of chickens. *National Institute of Animal Health Quarterly, Tokyo* **6**, 136.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurements with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265.

LUFT, J. H. (1961). Improvements in epoxy resin embedding methods. *Journal of Biophysical and Biochemical Cytology* **9**, 409.

MANILOFF, J., MOROWITZ, H. J. & BARRNETT, R. J. (1965). Ultrastructure and ribosomes of *Mycoplasma gallisepticum*. *Journal of Bacteriology* **90**, 193.

NE'EMAN, Z., KAHANE, I., KOVARTOVSKY, J. & RAZIN, S. (1972). Characterization of the mycoplasma membrane proteins. III. Gel filtration and immunological characterization of *Acholeplasma laidlawii* membrane proteins. *Biochimica et Biophysica Acta* **266**, 255.

OGRA, M. S. & BOHL, E. H. (1970). Growth inhibition test for identifying *Mycoplasma meleagridis* and its antibody. *Avian Diseases* **14**, 364.

RAZIN, S. (1963). Osmotic lysis of mycoplasma. *Journal of General Microbiology* **33**, 471.

RAZIN, S. (1973). The physiology of mycoplasmas. In *Advances in Microbial Physiology* (ed. A. H. Rose and D. W. Tempest). **10**, pp. 1–80. London: Academic Press.

RAZIN, S., ARGAMAN, M. & AVIGAN, J. (1963). Chemical composition of mycoplasma cells and membranes. *Journal of General Microbiology* **33**, 477.

RAZIN, S., KAHANE, I. & KOVARTOVSKY, J. (1972). Immunochemistry of mycoplasma membranes. In *Pathogenic Mycoplasmas*, A Ciba Foundation Symposium, p. 93. Amsterdam: Associated Scientific Publishers.

RAZIN, S., MOROWITZ, H. J. & TERRY, T. M. (1965). Membrane subunits of *Mycoplasma laidlawii* and their assembly to membrane-like structures. *Proceedings of the National Academy of Sciences, U.S.A.* **54**, 219.

REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208.

RICE, C. E. (1947). Atypical behaviour of certain avian antisera in complement-fixation tests. *Canadian Journal of Comparative Medicine* **11**, 236.

ROBERTS, D. H. (1969). Serological response produced in chickens by three strains of *Mycoplasma gallisepticum*. *Journal of Applied Bacteriology* **32**, 395.

ROTTEM, S., HASIN, M. & RAZIN, S. (1973). Binding of proteins to mycoplasma membranes. *Biochemica et Biophysica Acta* **298**, 876.

ROTTEM, S. & RAZIN, S. (1972). Isolation of mycoplasma membranes by digitonin. *Journal of Bacteriology* **110**, 699.

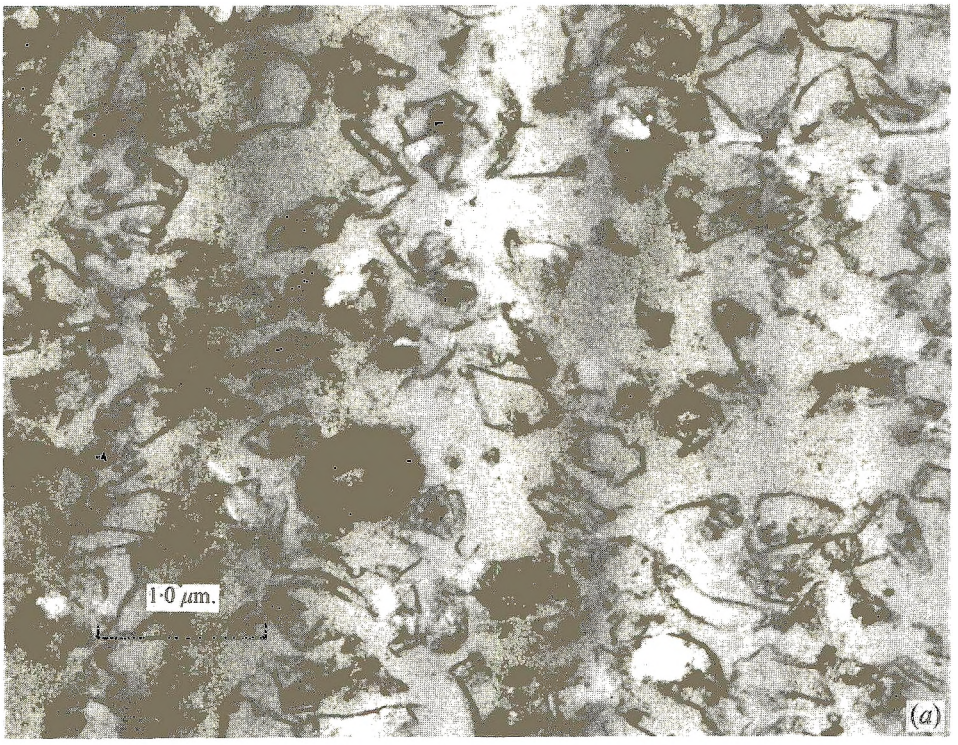
ROTTEM, S., STEIN, O. & RAZIN, S. (1968). Reassembly of mycoplasma membranes disaggregated by detergents. *Archives of Biochemistry and Biophysics* **125**, 46.

SMITH, P. F. & ROTHBLAT, G. H. (1960). Incorporation of cholesterol by pleuropneumonia-like organisms. *Journal of Bacteriology* **80**, 842.

TAYLOR-ROBINSON, D. & BERRY, D. M. (1969). The evaluation of the metabolic-inhibition technique for the study of *Mycoplasma gallisepticum*. *Journal of General Microbiology* **55**, 127.

TAYLOR-ROBINSON, D., PURCELL, R. H., WONG, D. C. & CHANOCK, R. M. (1966). A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. *Journal of Hygiene* **64**, 91.

WILLIAMS, M. H. & TAYLOR-ROBINSON, D. (1967). Antigenicity of mycoplasma membranes. *Nature, London* **215**, 973.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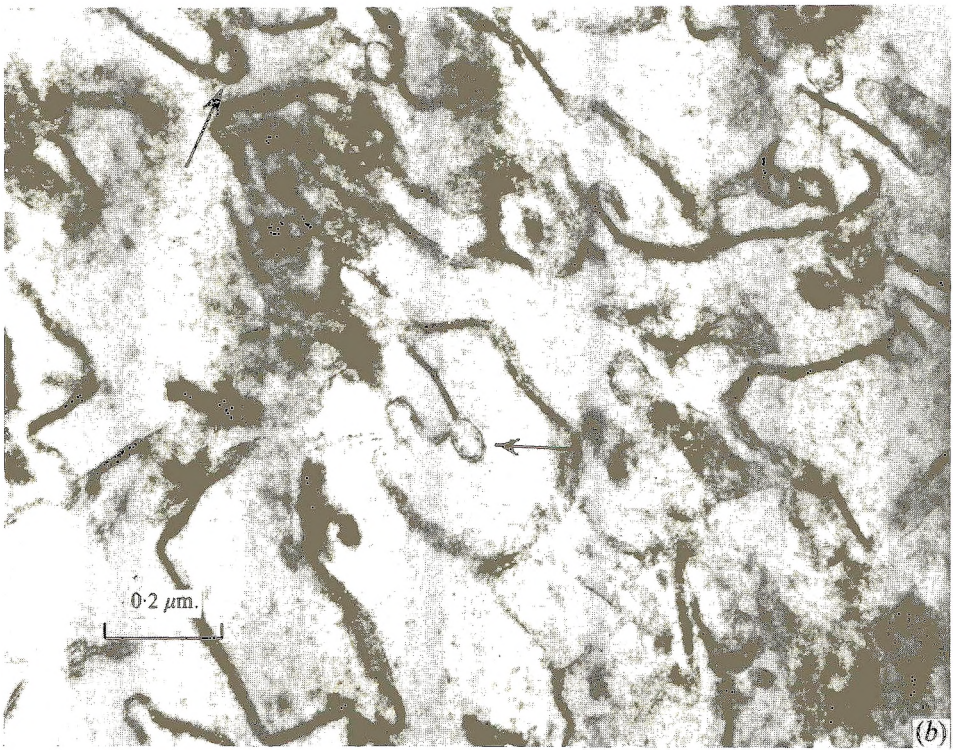
(a)


0·2 μm.
(b)

EXPLANATION OF PLATE

Thin sections of *M. gallisepticum* membranes isolated by digitonin. (a) Low magnification, showing also an unlysed cell. (*b*) High magnification, showing the characteristic trilaminar structure of the membranes and the vesicular extensions marked by arrows.

J. Hyg., Camb. (1973), **71**, 739
Printed in Great Britain

A note on the hygiene of meat mincing machines

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(*Received* 14 *March* 1973)

SUMMARY

Two mincing machines were cleaned by different methods, i.e. (*a*) a detergent/sterilizer method and (*b*) scrubbing parts in boiling (98·8° C.) water. Initial results indicated that, on reassembly, post-treatment contamination took place. Efforts to clean each machine as consisting of two distinct parts, (*a*) the casing and (*b*) removable parts, were more satisfactory. Four other mincers which could be completely dis-assembled were satisfactorily cleaned, but only in terms of percentage organisms surviving and not in terms of actual numbers surviving.

INTRODUCTION

No published data are available on the cleaning and maintenance of meat-mincing machines (B. C. Hobbs, personal communication) despite the wide range of bactericidal agents (detergents and detergent/sterilizers) which are marketed commercially for use in the food-processing industry (Thomas, 1969). Results on the cleaning and sterilizing of slicing machines, carving knives and can-openers and the possibility of cross-contamination by slicing machines and cleaning cloths have already been published (Gilbert & Maurer, 1968; Gilbert, 1969). However, much less published information is available on the cleaning of other meat-processing equipment.

This work resulted from a request from a 'carcase service laboratory' for an efficient method of cleaning large commercial meat mincers. The results can be applied to similar equipment in the meat industry, e.g. the sausage department of meat-processing firms, mincers in retail shops and 'home' mincing equipment.

MATERIALS AND METHODS

At first, two mincers, A and B, model No. RKW 82, Wexio Metaniska Verkstad, Vaxjo, Sweden, were available. After a normal day's use they were cleaned by the staff responsible for their maintenance. The parts were dismantled (mincer barrel, worm, blade, 0·95 cm. mincing plate, screw ring and feed tray) and each individually washed in a 2% (w/v) solution of Duet (Diversay, Ireland Ltd) at 71·1° C. (160° F.) and rinsed in clean water. The machine itself, (the feed funnel and worm-housing sleeve) was washed with a cloth in a warm (48·8° C.) silicate detergent solution and also rinsed in clean water. The parts were reassembled. The mincers were rinsed through with 500 ml. of quarter-strength Ringer's solution immediately after use and again after cleaning. The Ringer's solution was

poured over the feed tray and allowed to run down the feed funnel into the worm housing and over the worm with the motor running. The rinse was collected through a sterile glass funnel into a 500 ml. sterile plasma bottle. Serial decimal dilutions were made in Ringer's solution with the addition of 0·1% peptone (Straka & Stokes, 1957) and pour plates made with Plate Count Agar (P.C.A., Oxoid) for colony counts at 22° C. (3 days incubation) and 37° C. (incubation for 1 day).

A further experiment was carried out with the various machine parts and 'stripped' casings treated as separate pieces of equipment. After use the parts were placed in a plastic bag and the rinse (500 ml. Ringer's solution) poured into it. The machine casing was rinsed without the motor running, as the worm was not in position, and the rinse collected as before. After the cleaning procedure, described previously, the parts and machines were rinsed and counts made on the rinses from parts and casings before and after cleaning. The 'stripped' machines (feed funnel and worm housing sleeve) were more closely examined. It was noted that, by scraping these surfaces, a film of hardened soil (comminuted meat and fat) could be removed, therefore the use of cloths for cleaning was discontinued and the surfaces scrubbed with soap-impregnated pads. The diameters of the feed funnel (6·25 cm.) and the casing (21·5 cm.) precluded any other scrubbing method being used. After scrubbing the surfaces were thoroughly washed with clean water. The final method adopted to clean these mincers was:

Parts	Machines
Visible dirt removed, washed in warm (48·8° C.) water and then boiled (98·8° C.) for 10 min.	Visible dirt scraped off, surfaces scrubbed with soap pads and rinsed in clean water.

A further four mincing machines, C, D, E and F, became available to carry out additional tests (in retail shops). Two were 'Crypto Peerless', Model A.D. 12 (North Circular Road, London, N.W. 10) and two were U.S. Berkel, Model E 222 (Berkel Inc., La Porte, Ind. 46350, U.S.A.). These were chosen because all parts could be dismantled. They differed from the Swedish-made models in this respect. The normal cleaning procedure in each shop was as follows:

Mincer	Procedure
C	(*a*) Remove adhering meat from surfaces (*b*) Steep parts in water (98·8° C.) and hand wash when temperature dropped to *ca.* 48° C.
D	(*a*) As C (*b*) Wash in water (82·2° C.) + an anionic detergent
E	(*a*) As C (*b*) Wash in a hot solution (60° C.) of sodium carbonate (2·5%, w/v)
F	(*a*) As C (*b*) Wash in a hot solution (64·4–71·1° C.) of sodium carbonate (1·25%, w/v). Dry with clean cloth

Table 1. *Plate counts at* 22° C. *of rinses taken from mincers A and B (parts* in situ *and routine cleaning)*

Trial no.	Mincer	Count/ml. Before cleaning	Count/ml. After cleaning	Survival (%)
1	A	1,775	9,900	Increase
	B	260	504	Increase
2	A	2,260	13,250	Increase
3	A	575	185,100	Increase
	B	6,450	1,720	26·7
4	A	865	58,800	Increase
	B	123,000	89,400	72·7

Table 2. *Plate counts at* 22° C. *from rinses of parts and casings (separately) of mincers A and B*

Mincer	Equipment	Count/ml. Before cleaning	Count/ml. After cleaning	Survival (%)
A	Parts	9,750	1,240	12·7
	Casing	4,650	6,500	Increase
B	Parts	77,650	1,395	2·44
	Casing	149,000	186,250	Increase

RESULTS

The bacterial counts of two commercial meat mincers, A and B, before and after different methods of cleaning are shown in Tables 1–3.

In Table 1 it is seen that, after cleaning, higher counts were recorded in five out of seven instances. This increase in bacterial count was obviously derived from the casing (feed funnel and worm housing sleeve), which could not be dismantled. It is suggested that the post-treatment rinse washed organisms off these surfaces or there was a 'carry-over' effect as a result of poor cleaning methods.

Table 2 shows the effect of cleaning the parts and the casings separately. In each case there was a considerable reduction in contamination on the parts (87% in mincer A and 98% in mincer B). However, the casings themselves remained a potent focus of contamination. This result tends to support the points already made, namely an increase in post-treatment count was due to either a 'washing off' effect or poor cleaning techniques. These points are confirmed by the data presented in Table 3, which show the results of five trials on both mincers after cleaning the parts and 'stripped' scrubbed machines separately and rinsing them after reassembly. The highest survival count was 14% (mincer A) and the lowest survival count was $< 0{\cdot}1\%$ (mincer B).

In Table 4 is presented the data on the cleaning of four meat-mincing machines (C–F) which can be completely 'stripped', i.e. all parts which come in contact with meat can be removed for cleaning. The mean percentage survival at 37° C. ranged from 2·25 to 4·96 and at 22° C. from 0·28 to 0·82.

Table 3. *Plate counts at* 22° C. *of rinses taken from mincers A and B before and after improved cleaning technique*

Trial no.	Mincer	Count/ml. Before cleaning	Count/ml. After cleaning	Survival (%)
1	A	353,500	163	0·04
2	A	1,940	39	2·03
	B	6,450	182	2·82
3	A	475	68	14·31
	B	183	8	4·63
4	A	13,400	60	0·44
	B	68,500	1,840	2·68
5	A	537,500	21,550	4·00
	B	5,000,000	39,500	< 0·01

Table 4. *Colony counts at* 37° *and* 22° C. *from rinses of mincers C, D, E and F before and after cleaning*

Mincer	Trial no.	37° C. Count/ml. Before cleaning	37° C. Count/ml. After cleaning	37° C. Survival (%)	22° C. Count/ml. Before cleaning	22° C. Count/ml. After cleaning	22° C. Survival (%)
C	1	580,000	2,800	0·48	12,700,000	53,000	0·42
	2	120,000	210	0·18	1,670,000	4,600	0·28
	3	6,000	330	5·50	690,000	3,000	0·44
	4	50,200	2,470	4·92	3,890,000	44,000	2·14
				2·84*			0·82*
D	1	3,200,000	43,000	1·35	5,000,000	410,000	0·82
	2	59,000	9,200	5·60	2,400,000	19,000	0·80
	3	6,700	40	0·59	540,000	2,900	0·54
	4	19,000	280	1·47	1,930,000	19,600	1·12
				2·25*			0·82*
E	1	30,000	110	0·37	700,000	690	0·10
	2	6,000	580	9·67	1,410,000	2,100	0·15
	3	61,000	8,500	3·94	1,960,000	49,000	2·50
	4	790,000	1,000	0·13	13,300,000	8,000	0·06
				3·78*			0·70*
F	1	100,000	7,500	7·50	15,000,000	13,800	0·10
	2	51,000	1,090	2·14	1,380,000	6,300	0·46
	3	85,000	2,570	3·03	9,400,000	11,300	0·12
	4	81,000	31,100	7·17	8,800,000	39,000	0·45
				4·96*			0·28*

* Mean values.

DISCUSSION

Meat-mincing machines in which only some parts can be dismantled must obviously be treated as two distinct pieces of equipment for cleaning purposes. Removable parts can be satisfactorily cleaned as shown in this experiment. However, those parts which cannot be removed — in this case the feed funnel and the worm housing sleeve (casing) – must be given special attention by what might be described as 'in-place cleaning'. The use of cloths for this task cannot be recommended as this results in recontamination. The Food Hygiene Code of Practice (1969) states that all meat equipment should be designed to eliminate the accumulation of dirt. Some mincers comply with this specification. For example, the 'back creep' of meat juices into the gear box is prevented on some machines by a juice escape valve under the drive hub. Other makes of machine are so designed that the entire worm housing can be removed by loosening wing-nuts, leaving only the motor housing, which makes no contact with meat at any time. Although the percentage survival in four mincers ranged only from 0·13 to 9·67 at 37° C. and 0·06 to 2·50 at 22° C., in terms of actual numbers of surviving micro-organisms the results are unsatisfactory. Thus the mean pretreatment colony count (37° C.) in mincer C was 189×10^3/ml. and the mean post-treatment count was $14{\cdot}5 \times 10^2$/ml. The results at 22° C. are even worse (mincer C); a mean pre-treatment count of $4{\cdot}7 \times 10^6$ and a post-treatment mean of 26×10^3/ml.

Freshly minced meat constitutes one of the most challenging of meat products for quality assurance and public health protection. Reports of retail products with counts $> 10^6$/g. denote microbial contamination that should be reduced or eliminated (Tiwari & Maxcy, 1972). It has been found that off-odours usually develop in minced meat by the time it contains about 10^8/g. (Pearson, 1970). The degree of contamination of a mincer will depend on the number of organisms in the raw meat and the higher the numbers the more difficult it will be to remove them by cleaning methods.

Recommendations

(1) Ensure that all parts which cannot be removed receive special attention by 'in-place' cleaning. Cloths cannot be recommended for this purpose. Mincers do not lend themselves easily to 'in-place' cleaning because of the inaccessability of parts. However, one method which has proved successful in our hands is to scrub such parts with soap pads.

(2) The design of such mincers should be modified so that all parts which come in contact with meat can be dismantled.

(3) Use a proved method of cleaning, either chemical (detergent or detergent/sterilizer) or physical (boiling parts in water). Do not rely on the bactericidal effect of any proprietary chemical which may lead to a false sense of security (see Gilbert & Maurer, 1968).

(4) Apply the chosen method daily or preferably twice daily.

(5) If possible, arrange to have frequent bacteriological rinse tests carried out (such a method is described in this paper) to ensure that only small numbers of micro-organisms survive, e.g. 100/ml. at 22° C.

The author wishes to record his appreciation for the help and co-operation of the management of the various retail shops. His grateful thanks are also due to Mr S. N. Reid, F.I.M.L.T., Miss C. Murphy and Mr B. Lynch for skilful technical assistance. Thanks are also due to Dr R. J. Gilbert for useful comments in the presentation of the paper.

REFERENCES

FOOD HYGIENE CODE OF PRACTICE (1969). No 8. *Hygiene in the Meat Trades*, parts I and II. London: H.M.S.O.

GILBERT, R. J. (1969). Cross-contamination by cooked-meat slicing machines and cleaning cloths. *Journal of Hygiene* **67**, 249.

GILBERT, R. J. & MAURER, I. M. (1968). The hygiene of slicing machines, carving knives and can openers. *Journal of Hygiene* **66**, 439.

PEARSON, D. (1970). Effect on various spoilage values of the addition of sulphite and chlortetracycline to beef stored at 5° C. *Journal of Food Technology* **5**, 144.

STRAKA, R. P. & STOKES, J. L. (1957). Rapid destruction of bacteria in commonly used diluents and its elimination. *Applied Microbiology* **5**, 21.

THOMAS, G. A. (1969). The selection of detergents and disinfectants for use in food plant cleaning. *British Food Manufacturing Industries Research Association, Technical Circular* no. 433.

TIWARI, N. P. & MAXCY, R. B. (1972). Comparative growth of salmonellae, coliforms and other members of the microflora of raw and radurized ground beef. *Journal of Milk and Food Technology* **35**, 455.

J. Hyg., Camb. (1973), **71**, 745
Printed in Great Britain

Effect of sub-lethal treatment with formalin on the germination of *Aspergillus fumigatus* spores

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(*Received* 16 *March* 1973)

SUMMARY

Sub-lethal exposure of *Aspergillus fumigatus* spore suspensions to formalin resulted in prolongation by 1–22 days of the period of less than one day normally needed by spores to produce visible growth in Sabouraud's liquid medium at 37° C.; the degree of delay depended on the concentration of formalin and the duration of exposure, and was due to an increase in the germination-time of spores. The formalin concentration could be adjusted so as to affect the germination-time of almost all spores in a suspension without reducing viability. The effect on germination was not abolished by thorough washing or treatment with sodium sulphite. The spores of four different strains of *A. fumigatus* and of cultures aged 3 to 14 days reacted similarly to formalin treatment. Although of greatly reduced virulence for mice, affected viable spores were still capable of producing infection and death following intravenous inoculation, provided they were not eliminated by the host before germination occurred.

INTRODUCTION

Unlike many other germicides, formalin is capable, even when considerably diluted, of destroying all forms of microbic life (Williams, Blowers, Garrod & Shooter, 1966). Nordgren (1939) reviewed much of the earlier literature. Müller (1920) considered that the action of formalin on anthrax spores was partially reversible by ammonia, but Gegenbauer (1921) was unable to confirm this. Hailer (1921*a*, 1921*b*) concluded that sodium sulphite was able to reverse the action of formaldehyde solution on anthrax spores and certain vegetative bacteria, but Nordgren (1939) preferred to leave this question open, although his experiments led him to accept that objects exposed to formaldehyde should be treated with sodium sulphite before being subjected to sterility tests. In experiments on disinfection by gaseous formaldehyde (Report, 1956) the treatment of material with sodium sulphite solution or the incorporation of sodium sulphite into culture media was sometimes helpful in removing free formaldehyde. Schultz & Gebhardt (1935) reactivated formaldehyde-treated bacteriophage merely by dilution, and Ross & Stanley (1938) found that dialysis at pH 3 reactivated formalin-inactivated tobacco-mosaic virus. Englesberg (1952) suggested that the large differences which he found in viable counts of formaldehyde-treated *Pseudomonas fluorescens* indicated reactivation by certain media, but Nash & Hirch (1954) offered the

alternative explanation that damaged organisms might require a better growth medium than undamaged organisms. They found that a mixture of dimedone and morpholine reactivated formalin-treated bacteria and ascribed the effect to interference with the lethal process rather than to reversal of some action which had proceeded to completion, or to simple neutralization of free formaldehyde.

The literature contains little information on the action of formaldehyde on fungi. The present report arises from tests of formalin on *Aspergillus fumigatus* spores, made as a prelude to experiments on immunization with dead vaccines. Certain striking effects on germination were demonstrated and the virulence of spores treated with sub-lethal doses of formalin was examined.

MATERIALS AND METHODS

Strains of A. fumigatus

Purified seed cultures of strains AF_1, AF_2, AF_3, AF_4 and AF_5 (Smith, 1972) were stored at $-20°$ C. until used.

Preparation of spore suspensions

Spores harvested from Sabouraud's dextrose-agar cultures (Oxoid, CM 41) grown for 3 days at 37° C. were triple-washed, suspended in nutrient broth (Oxoid, CM 67) and counted, as described by Smith (1972).

Formalin-treatment of spore suspensions

Dilutions of formalin in distilled water were added to spore suspensions in the proportion 1:9 (v/v) to produce the required final concentration. A similar proportion of sterile distilled water alone was added to the control spore suspensions. The original formalin was stated by the manufacturers to contain 37 to 41 % w/v formaldehyde and 11 to 14 % methanol. Treatment with formalin was carried out at 4° C. with constant mechanical agitation.

Examination of viability and delayed germination of formalin-treated spores

Unless stated otherwise, Sabouraud's dextrose liquid medium (Oxoid, CM 147), pH 5·7 approximately, was dispensed in 10 ml. volumes in 25 ml. McCartney bottles and inocula consisted of small standard loopfuls of spore suspension; the standard loop used throughout the experiments was made from 23 SWG platinum and had an internal diameter of approximately 2 mm. Thus, any residual free formalin was diluted to an ineffective level. To permit the entry of sufficient air for complete growth of *A. fumigatus*, the bottles were closed either with loose metal caps or cotton-wool stoppers. The incubator atmosphere was humidified during protracted incubation at 37° C. to reduce the rate of evaporation of culture medium. In one experiment sodium sulphite was added to autoclaved Sabouraud's liquid medium, which was then re-sterilized by filtration. In any experiment, cultures were examined at least once a day for the first appearance of visible growth, and incubation of negative cultures was continued for at least 2 weeks after the last positive

culture was recorded. As shown later, delays in the time taken for visible growth to occur were due to increases in germination time.

Mice

Females weighing 20 g. were obtained from the outbred, closed colony of Swiss white mice referred to by Smith (1972) as the SA colony. Spores suspended in nutrient broth were inoculated intravenously in 0·25 ml. doses.

RESULTS

Effect on germination of exposing spore suspension to different concentrations of formalin for different times

A spore suspension prepared from strain AF_1 was treated for 11 days with concentrations of formalin ranging from 0·05 to 1·8 % (equivalent to approximately 0·02 to 0·72 % formaldehyde). After each day of treatment, a small standard loopful from each bottle including that containing untreated control suspension was cultured in Sabouraud's liquid medium. Viable counts of the control suspension made at the beginning and end of the 11-day period of treatment and on one intermediate occasion showed that the number of living spores remained constant throughout at 139–178 million per ml.

Table 1 shows that each daily subculture of the control suspension first produced visible growth within 24 hr. incubation, but formalin induced a delay the length of which usually increased as the concentration of formalin and the duration of exposure increased. The longest delay produced in this experiment was 19 days. Once visible growth appeared, it proceeded at the normal rapid rate, and in similar experiments repeated microscopical examination of formalin-treated suspensions revealed only ungerminated spores up to the day before the first appearance of visible growth. Thus, the effect of the formalin was on the germination-time of the spores.

Treatment with 1·8 % formalin for 24 hr. failed to sterilize, but 0·4 % produced sterility after 7 days' treatment. Although some evaporation of the medium had occurred by the time the experiment was terminated, each negative bottle readily supported growth from an inoculum of 0·1 ml. medium containing approximately eight spores. This showed that concentration of the constituents of the medium, including the small amount of free formalin, which occurred during prolonged incubation had no appreciable inhibitory effect on growth.

A further experiment showed that the action of formalin on washed, saline suspensions of spores was similar to that on nutrient broth suspensions.

Quantitative examination of the effect on germination-time and viability of exposing AF_1 spore suspension to different concentrations of formalin for 24 hr.

A preliminary experiment is briefly described, as it assists in interpreting certain aspects of the main experiment. Approximately 18 spores produced normal growth in 4 ml. Sabouraud's liquid medium containing 0·0016 % formalin, but no macro-

Table 1. *Effect of formalin on the germination of strain AF_1 spores*

Duration of exposure to formalin (days)	Time taken (days) for visible growth to occur from subcultures of spore suspension exposed to formalin in concentrations (%) of											
	0	0·05	0·1	0·2	0·4	0·6	0·8	1·0	1·2	1·4	1·6	1·8
1	1	1	1	2	3	5	6	7	10	12	14	15
2	1	1	1	2	4	7	11	10	N	N	N	N
3	1	1	2	3	6	12	N	N	N	N	N	N
4	1	1	2	3	8	N	N	N	N	N	N	N
5	1	1	2	4	11	N	N	N	N	N	N	N
6	1	1	3	6	20	N	N	N	N	N	N	N
7	1	2	3	6	N	N	N	N	N	N	N	N
8	1	2	3	8	N	N	N	N	N	N	N	N
9	1	2	4	9	N	N	N	N	N	N	N	N
10	1	2	3	10	N	N	N	N	N	N	N	N
11	1	2	2	13	N	N	N	N	N	N	N	N

N = no growth.

Table 2. *Quantitative examination of the effect of formalin on germination-time in relation to viability of strain AF_1 spores*

Number of living or dead spores in 9 ml. liquid medium	Time taken (days) for visible growth to occur from decimal dilutions of a spore suspension exposed for 24 hr. to formalin in concentrations (%) of					
	0	0·2	0·6	1·0	1·4	1·8
152×10^6	1	3	N*	N†	N†	N†
152×10^5	1	2	4	12	N	N
152×10^4	1	2	4	3	15	N
152×10^3	1	2	5	9	17	N
152×10^2	1	2	5	10	23	N
152×10	1	2	5	10	N	N
152	1	2	5	11	N	N
15	1	2	6	N	N	N
1–2	1	N	6	N	N	N

N = no growth, but at the end of the experiment, inoculation with 0·1 ml. medium containing nine *A. fumigatus* spores gave normal growth on incubation.

N* = no growth, and medium produced mycelial growth only from nine spores inoculated at the end of the experiment.

N† = no growth, and medium failed to produce growth from nine spores inoculated at the end of the experiment.

scopic growth in the same medium containing 0·04 % formalin. Mycelium and surface spores were produced in the presence of 0·008 % formalin, but growth was slightly retarded.

In the main experiment (Table 2) a spore suspension prepared from strain AF_1 was treated for 24 hr. with five different concentrations of formalin ranging from 0·2 to 1·8 %. A series of decimal dilutions, ranging from 10^{-1} to 10^{-9} and prepared by transferring 1 ml. volumes of suspension into 9 ml. volumes of Sabouraud's

liquid medium, was made from each of the five bottles containing formalin-treated spores, and also from a control bottle containing untreated spores. In addition, a viable count of the control portion of the spore suspension was made by the plate method (Smith, 1972), thus enabling the number of spores, alive or dead, in the limiting dilution of the six series to be calculated. The 54 dilution bottles were incubated at 37° C., and the times of first appearance of visible growth as revealed by daily examination are shown in Table 2.

Even 1–2 spores from the control suspension produced visible growth within 24 hr. Formalin in low concentrations induced only a delay in germination, but in higher concentrations it also had a sporicidal effect; a concentration of 1·8 % produced complete sterilization.

Treatment with 0·6 % formalin had no effect on viability, but the delays in production of visible growth from spores were distributed as follows: not more than 1 in 10 spores was affected for less than 5 days; not more than 1 in 10^5 for less than 4 days; not more than 1 in 10^6 for less than 3 days. It is more than likely that the number of spores unaffected by 0·6 % formalin – if such spores existed at all – was considerably less than 1 in 10^6, but a firm statement cannot be made because the bottle containing 152×10^5 spores (Table 2) may have contained enough free formalin to exert some slight effect. Between 1 in 10^3 and 1 in 10^4 spores survived treatment with 1·4 % formalin, but of the survivors at least 99 % had a germination-time which was prolonged by 14–22 days.

Table 2 shows that at least four and probably five bottles containing 10^{-1} dilutions of spore suspension (152×10^6 spores) possessed sufficient free formalin to prevent growth or affect it adversely. It also appears that free formalin carried over into the 10^{-2} dilution of spore suspension treated with 1·0 % formalin played some part in delaying the appearance of growth; this should not be confused with the phenomenon with which this paper is mainly concerned – i.e. delayed germination in the absence of an effective concentration of free formalin.

Effect of formalin on four additional strains of A. fumigatus

Spore suspensions from strains AF_2, AF_3, AF_4 and AF_5 were adjusted to the opacity of Brown's tube 20, treated with 0·6 % formalin for 3 days, and subcultured in Sabouraud's liquid medium with a small standard loop after each day of treatment. Untreated control suspensions were also subcultured. Table 3 shows that the effect of 0·6 % formalin on germination of spores from these four additional strains was similar to that already found with strain AF_1.

Effect of formalin on spores from cultures of different ages

Spore suspensions from 7-day-old and 14-day-old cultures of strain AF_1 were prepared, formalinized and subcultured by the method described in the previous experiment. After treatment with 0·6 % formalin for 1, 2 and 3 days, the spores from 7-day culture took 5, 6 and 13 days respectively to produce visible growth in subcultures, as compared with less than 1 day for control spores. The corresponding results for spores from 14-day culture were 5, 7 and 13 days. These figures were similar to those obtained with spores from 3-day-old cultures (Tables 1 and 3).

Table 3. *Effect of formalin on the germination of spores of strains AF_2, AF_3, AF_4 and AF_5*

Duration of exposure to formalin (days)	Time taken (days) for visible growth to occur from subcultures of spore suspensions prepared from four strains of *A. fumigatus*							
	AF_2		AF_3		AF_4		AF_5	
	C	F	C	F	C	F	C	F
1	1	3	1	6	1	5	1	5
2	1	7	1	10	1	9	1	10
3	1	11	1	18	1	13	1	13

Spore suspensions with the opacity of Brown's tube 20 were treated with 0·6% formalin. C, control suspension; F, formalin-treated suspension.

Failure of thorough washing to abolish the effect of formalin on germination-time of spores

Spore suspension prepared from strain AF_1 and adjusted to the opacity of Brown's tube 20 was treated with 0·6% formalin for 24 hr., and an equal volume was used as control suspension. Both the formalinized and control suspensions were washed six times by centrifugation and resuspension in 10 ml. volumes of nutrient broth with thorough agitation. Subculture showed that the production of visible growth from control and formalin-treated spores took 1 and 5 days respectively. Comparison with the data in Tables 1 and 3 indicates that the washing procedure had no significant effect.

Failure of sodium sulphite treatment to abolish the effect of formalin on germination-time of spores

A suspension of strain AF_1 spores was made to the opacity of Brown's tube 28. Control spore suspension and suspension treated with 0·6% formalin for 24 hr. were subcultured by means of a standard loop in 10 ml. volumes of Sabouraud's liquid medium, and in the same medium containing five doubling concentrations of sodium sulphite ranging from 0·25 to 4·0%. Cultures of the formalin-treated spores in each of the six media were made in groups of six replicates.

Subcultures of control spores invariably gave visible growth within 24 hr., although sodium sulphite had an obvious inhibitory effect, varying from just appreciable to almost complete in 0·5% and 4·0% concentrations, respectively. Formalin-treated spores in medium without sodium sulphite gave rise to visible growth in 3 days. This time was increased by the presence of sodium sulphite; it varied from 4–6 days in a 0·25% concentration to 7–9 days in a 4·0% concentration.

Thus the experiment gave no evidence that sodium sulphite could reverse the effect of formalin on spores. If any such tendency existed, it was too slight to reveal itself in the presence of the anti-fungal effect of the sodium sulphite.

Table 4. *Reduction in virulence of spores by sub-lethal treatment with formalin*

Days after inoculation	Deaths in mice following intravenous inoculation with control spores in doses of			formalinised spores in doses of		
	$51{\cdot}2 \times 10^6$	$12{\cdot}8 \times 10^6$	$3{\cdot}2 \times 10^6$	$51{\cdot}2 \times 10^6$	$12{\cdot}8 \times 10^6$	$3{\cdot}2 \times 10^6$
7	12/12	12/12	1/12	0/6	0/6	0/6
16	—	—	3/12	2/6	0/6	0/6
28	—	—	5/12	2/6	1/6	1/6
			(5/7)	(2/4)	(1/5)	(0/5)

Suspension containing 205×10^6 spores per ml. was treated with 0·6% formalin for 24 hr. Figures in parentheses indicate number of survivors showing active renal aspergillosis when killed 28 days after inoculation.

Virulence of spores after sub-lethal treatment with formalin

A suspension containing 205×10^6 viable spores per ml. was prepared from strain AF_1. A portion was treated for 24 hr. with 0·6% formalin whilst a second portion was used as control suspension. The formalin-treated and control suspensions were then thoroughly washed six times and resuspended to the original volumes in nutrient broth. Decimal dilutions of control and treated suspensions ranging from 10^{-1} to 10^{-10} were made in Sabouraud's liquid medium and incubated at 37° C.; the time of first appearance of growth in each bottle was recorded. Even 1–2 control spores produced visible growth within 24 hr. The formalin treatment was shown not to have killed any spores, but delays in production of visible growth from spores were distributed as follows: not more than 1 in 10^2 spores, 1 in 10^4 spores and 1 in 10^6 spores were affected for less than 5 days, 4 days and 1 day respectively. Less than 1 in 10^6 but more than 1 in 10^7 were not appreciably affected by the formalin.

Six groups of mice were inoculated intravenously with doses of (millions) 51·2, 12·8 and 3·2 of the freshly prepared formalin-treated or control spores. The mortality patterns and the number of survivors found to be infected when killed 28 days after inoculation are shown in Table 4. It is clear that spores whose germination-time was increased by formalin treatment were of greatly reduced virulence. Nevertheless, reference to the data given by Smith (1972) on the MID and MLD of strain AF_1 for SA mice leads to the following conclusion: the small number of infections and deaths which did occur in mice given formalin-treated spores could not have resulted from the minute proportion of the inocula (< 1 in 10^6 spores) which remained unaffected by the formalin. Thus, spores whose germination-time was increased by formalin treatment were still capable of producing disease, provided they were not eliminated by the host's defence mechanisms before germination occurred.

DISCUSSION

In relating these findings to earlier work on formaldehyde the important differences between bacterial endospores and the phialospores (conidia) of *A. fumigatus* should be borne in mind.

Fungal spores can remove large quantities of certain fungicides from dilute solutions, often accumulating up to 1·0 % of their own weight (Miller, McCallan & Weed, 1953; Byrde, 1966; Somers, 1966). Müller & Biedermann (1952) showed that germination of *Alternaria tenuis* spores was retarded by sub-lethal doses of copper, but that the spores later recovered; retained copper could be removed by exchange and chelation, with restoration of germination potential. Fildes (1940) found that the anti-bacterial action of mercury was reversible by -SH compounds.

The delaying effect of certain sub-lethal concentrations of formalin on the germination of *A. fumigatus* spores was a phenomenon quite distinct from the inhibition of germination and growth which may result from the carry-over of free formalin into subcultures. It was noted not only when subcultures were made by a method which diluted any free formalin to an ineffective level, but also in formalin-treated spores that had been thoroughly washed. It could not be reversed by treatment with sodium sulphite, but protracted incubation at 37° C. in Sabouraud's liquid medium eventually restored the ability to germinate after delays which varied from 1 to 22 days according to the duration of exposure to formalin and the concentration used. A particular degree of formalin treatment was capable of delaying the germination of almost all spores in a suspension, without reducing viability. In testing for sterility of formalin-treated suspensions of *A. fumigatus* spores, it is important to appreciate that subcultures should be incubated for at least a month. Although fungal spores of different ages may vary in their susceptibility to fungicides (McCallan, 1930), *A. fumigatus* spores from cultures aged 3, 7 and 14 days did not differ significantly in respect of the effect of formalin on germination-time.

A. fumigatus spores whose germination-time had been prolonged by treatment with sub-lethal concentrations of formalin were of greatly reduced virulence for mice, yet were still capable of producing infection and death. It seems possible that their ability to produce disease was completely unaffected by formalin, provided that they were not eliminated by the host's defence mechanisms before germination occurred.

I am grateful to Mr M. R. Fennellow and Miss Miriam Wood for invaluable technical assistance.

REFERENCES

BYRDE, R. J. W. (1966). The vulnerability of fungus spores to fungicides. In *The Fungus Spore*, Proceedings of the eighteenth symposium of the Colston Research Society (ed. M. F. Madelin), p. 289. London: Butterworths.

ENGLESBERG, E. (1952). The mutagenic action of formaldehyde on bacteria. *Journal of Bacteriology* **63**, 1.

FILDES, P. (1940). The mechanism of the anti-bacterial action of mercury. *British Journal of Experimental Pathology* **21**, 67.

GEGENBAUER, V. (1921). Studien über die Desinfektionswirkung wässeriger Formaldehydlösungen. *Archiv für Hygiene* **90**, 239.

HAILER, E. (1921*a*). Versuche über die Beziehung zwischen Formaldehyd und der Bakterien- und Sporenzelle. *Biochemische Zeitschrift* **125**, 69.

HAILER, E. (1921*b*). Die baktericide Nachwirkung von Formaldehydlösungen. *Biochemische Zeitschrift* **125**, 84.

MCCALLAN, S. E. A. (1930). Studies of fungicides II. Testing protective fungicidesin the laboratory. *Memoirs. Cornell University Agricultural Experiment Station* **128**, 8.

MILLER, L. P., MCCALLAN, S. E. A. & WEED, R. M. (1953). Rate of uptake and toxic dose on a spore weight basis of various fungicides. *Contributions. Boyce Thompson Institute for Plant Research* **17**, 173.

MÜLLER, A. (1920). Die Resistenz der Milzbrandsporen gegen Chlor, Pickelflussigkeit, Formaldehyd und Sublimat. *Archiv für Hygiene* **89**, 363.

MÜLLER, E. & BIEDERMANN, W. (1952). Der Einfluss von Cu^{+6}-Ionen auf den Keimungsablauf von *Alternaria tenuis*. *Phytopathologische Zeitschrift* **19**, 343.

NASH, T. & HIRCH, A. (1954). The revival of formaldehyde-treated bacteria. *Journal of Applied Chemistry, London* **4**, 458.

NORDGREN, G. (1939). Investigations on the sterilization efficacy of gaseous formaldehyde. *Acta pathologica et microbiologica scandinavica. Supplementum* XL.

REPORT (1956). Disinfection of fabrics with gaseous formadehyde. Report by the Committee on Formaldehyde Disinfection of the Public Health Laboratory Service. *Journal of Hygiene* **56**, 488.

ROSS, A. F. & STANLEY, W. M. (1938). The partial reactivation of formolized tobacco mosaic virus protein. *Journal of General Physiology* **22**, 165.

SCHULTZ, E. W. & GEBHARDT, L. P. (1935). Nature of formalin inactivation of bacteriophage. *Proceedings of the Society for Experimental Biology and Medicine* **32**, 1111.

SMITH, G. R. (1972). Experimental aspergillosis in mice: aspects of resistance. *Journal of Hygiene* **70**, 741.

SOMERS, E. (1966). The sites of reaction of fungicides in spores. In *The Fungus Spore*, Proceedings of the eighteenth symposium of the Colston Research Society (ed. M. F. Madelin), p. 299. London: Butterworths.

WILLIAMS, R. E. O., BLOWERS, R., GARROD, L. P. & SHOOTER, R. A. (1966). In *Hospital Infection. Causes and Prevention*, 2nd ed. London: Lloyd-Luke (Medical Books) Ltd.

J. Hyg., Camb. (1973), **71**, 755
With 1 plate
Printed in Great Britain

Skin tests with influenza virus

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(*Received* 26 *March* 1973)

SUMMARY

Skin reactions have been produced in normal subjects by the injection of highly purified influenza A and B viruses. The reactions reached a maximum at 24–48 hr. and the histological pattern was compatible with a delayed hypersensitivity reaction. There was no close relation between skin test results and circulating antibodies. Twenty-seven subjects were challenged intranasally with attenuated influenza A (H3N2): 5 of 14 skin-test-negative subjects were infected, but none of 13 skin-test-positive subjects.

INTRODUCTION

The presence of haemagglutination-inhibiting antibody in serum has been known for a long time to indicate resistance to infection with the influenza virus (Hobson, Beare & Gardner, 1971). However, in recent studies it has not proved possible to account completely for the resistance of volunteers to infection with live influenza virus vaccines by the presence of antibody against either of the surface components of the virus, i.e. haemagglutinin (HA) or neuraminidase (N), whether this was measured in sera or nasal secretions (Freestone *et al.* 1972) It therefore seemed reasonable to ask whether cell-mediated immunity might play a part, and we decided to start by investigating the skin reactions to the virus, which were reported many years ago (Beveridge & Burnet, 1944; Beveridge, 1952).

MATERIALS AND METHODS

Volunteers

The subjects were 41 male and 52 female members of the staff of this institute, aged between 18 and 66 years, who volunteered to take part after the nature of the procedures and the objectives of the investigation had been explained to them by one of us. The whole project had been previously reviewed and approved by the Ethical Committee of the Northwick Park Hospital.

Antigens

Influenza viruses were propagated in the allantoic cavity of chick embryos and purified by zonal ultracentrifugation. They were prepared and safety-tested as for influenza vaccine production, being inactivated with formaldehyde and stored at 4° C. in 1·2% sucrose. Immediately before use these antigens were diluted in

isotonic saline. Three batches of antigens were used, two being prepared from A2/Aichi/68 (H3N2) and B/Mass/3/66 by Richardson Merrell Laboratories, and one from A/Hong Kong/1/68X (H3N2) by Evans Medical Ltd. A control fluid was prepared from allantoic fluid from uninoculated eggs. This was diluted to the same protein concentration as the skin test antigen.

Antibody measurements

Haemagglutination inhibition (HI) tests were performed by a standard method (Tyrrell, Peto & King, 1967).

Skin tests

In all cases the materials were inoculated in 0·1 ml. volumes into the skin of the volar aspect of the forearm. The skin was examined and any erythema or swelling was measured, usually after 18–24 hr. but in early experiments at shorter and longer intervals than this.

Histology

Typical lesions were excised under local anaesthesia after 24 hr. The tissue was divided into two portions. One part was snap-frozen with liquid nitrogen and cryostat sections cut. These were stained by immunofluorescent techniques for viral antigen, immunoglobulins and complement. The remaining tissue was fixed in formalin and paraffin sections examined after staining with haematoxylin and eosin, azur A and methyl green–pyronin.

RESULTS

The injection site was examined repeatedly during the first 5 days. Reactions only became apparent after some hours and usually reached a peak about 24 hr. after inoculation. They declined somewhat at 48 hr. and then continued for several further days. They seemed to be specific. For example, in a series of tests using 4 CCA units of antigen, reactions were seen in 12 of 42 subjects against influenza A, and 17 of 42 subjects against influenza B; there were no reactions to the control fluid apart from one mild immediate type reaction. Eleven subjects reacted to only one antigen (Table 1), but there was an excess of subjects reacting to both, perhaps because some developed skin reactivity more readily than others. Repeated tests were fairly reproducible in size; the smallest significant reaction was arbitrarily taken as one over 5 mm. in diameter and this is referred to as 'positive' later in this paper. There was a relation between the amount of antigen administered and the reaction observed; thus subjects who failed to react to 4 units might react to a larger dose, while those who did react to 4 usually had a larger reaction to a larger dose (Fig. 1).

Histology

Biopsies of typical lesions due to both influenza A and B were examined. They showed considerable infiltration with lymphocytes and mononuclear cells aggregated mainly around small blood vessels but with a lesser infiltrate about sweat glands and skin appendages (Plate 1). Only scanty mast cells were noted. Cryostat

Table 1. *Results of skin tests in 42 subjects each tested with both A and B antigens using 4CCA units*

		Tested with A antigen	
		No. skin-test positive	No. skin-test negative
Tested with B antigen	No. skin-test positive	9	8
	No. skin-test negative	3	22

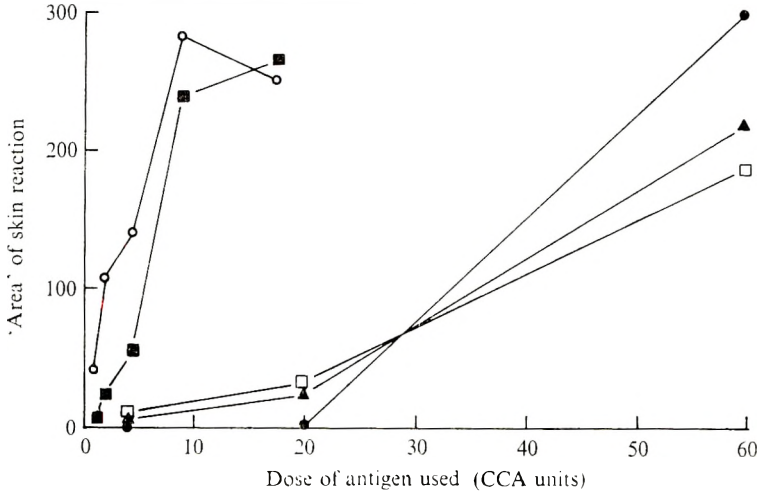


Fig. 1. Relation between amount of antigen injected and size of skin lesion at 24 hr., expressed as the product of the greatest and least diameters ('area'). Five subjects tested with A2/Aichi/68.

sections examined for immunoglobulin and complement components gave negative results. We consider that the histological and immunological features indicated a delayed type reaction and were not compatible with an Arthus reaction.

Relationship to circulating antibodies

Blood collected from volunteers at the time of testing was titrated for antihaemagglutinin antibodies by the HI test, and these results are shown in Fig. 2. It can be seen that there were few volunteers with low titres of antibody who also had positive skin reactions, although there were substantial numbers of subjects with high titres of antibody who nevertheless had negative skin reactions.

The tests were repeated using radial immunodiffusion (Schild, Henry-Aymard & Pereira, 1972) with an H3N2 virus as antigen. This technique measures antibodies against both neuraminidase and haemagglutinin. The test was not quite so sensitive, but again (Fig. 3) there was no close relationship with the results of skin tests. Eleven of 27 subjects seronegative by this test had positive skin tests, while 9 of 23 seropositive subjects had positive skin tests. These data again discount the possibility that the skin lesions are those of an Arthus reaction.

Relation to infection

We assumed that skin sensitivity must arise as a result of exposure to an antigen related to the one used in the test and tried to find out what type of stimulus would induce skin sensitivity.

Table 2. *Response to vaccine challenge*

	Initial reciprocal HI titre	No. infected	Total
Skin-test positive	<20	0/0	0/13
	$\geqslant 20$	0/13	
Skin-test negative	<20	2/3	5/14
	$\geqslant 20$	3/11	

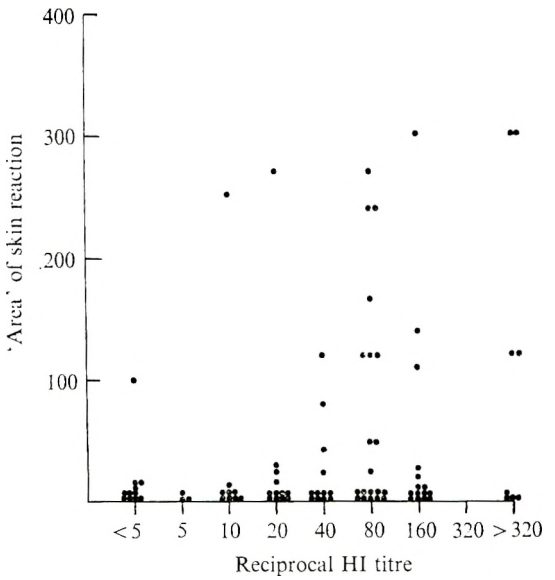


Fig. 2. Relation between the 'area' of skin reactions to H3N2 strain and the serum haemagglutination inhibiting antibody titre. Results in 83 subjects. Those with lower titres have less reactions, but there are reactions in some with very low titres.

Seven volunteers were inoculated intramuscularly with a standard dose of killed vaccine prepared from A2/Hong Kong/68 (H3N2) virus, and three developed a substantial rise in circulating HI antibodies. Nevertheless there was no significant change in skin reactivity. Thinking that infection of the respiratory tract might be needed, other volunteers were given as an intranasal spray 10^6 infectious doses of an attenuated live influenza virus vaccine. Of 27 volunteers five became infected as judged by an HI antibody response, but again there was no significant increase or decrease in skin reactivity when they were tested 2 weeks after inoculation. Thinking that the infection might have been too mild, we also tested five patients who had just recovered from clinical influenza due to laboratory-confirmed infection with an H3N2 virus of serotype A2/Eng/42/72; none had positive skin tests.

We also considered that a positive skin test might be associated with resistance to infection. We therefore analysed the outcome of giving live vaccine to volunteers according to the skin test results and antibody titres. Table 2 shows that whether the volunteer had antibody or not, the infection always occurred in subjects who were skin-test-negative; and the overall differences reach statistical significance.

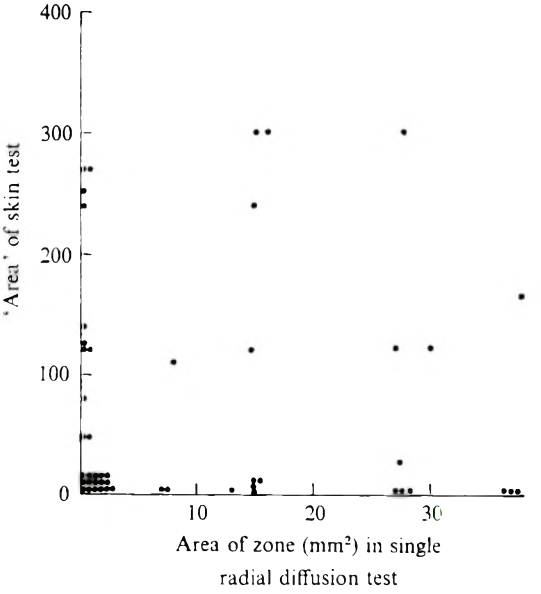


Fig. 3. Relation between 'area' of skin reaction and antibody against both HA and N of H3N2 as measured by radial immunodiffusion. Results in 50 subjects. Relationship with antibody concentration is less obvious.

DISCUSSION

Using purer antigens than earlier workers, we have again produced skin reactions to influenza virus. All the evidence – i.e. the timing, the histological appearances, the lack of relation with circulating antibody and the absence of immunoglobulins or complement in the lesion – points to this being a delayed type reaction due to cell mediated immunity. The specificity of the reactions shows that they are due to a particular viral component, but further work is necessary to determine whether they can be produced by pure haemagglutinin or other peptides extracted from virus particles. In addition, the mechanism of the reaction should be studied; for example, by looking for specific transformation of lymphocytes with virus antigen, as was done in a different context by Denman *et al.* (1970). This approach would make it possible to look for blocking antibodies, the presence of which might account for the existence of at least some of those subjects who have clearly been exposed to virus because they have serum antibody and who resist reinfection but who have negative skin reactions. Waldman & Henney (1971) have shown in animals that there may be separate populations of virus-sensitive lymphocytes, in that those derived from the respiratory tract are more readily transformed after vaccination by the respiratory route than splenic lymphocytes, while the latter are more sensitive after parenteral vaccination. It seems important now to decide whether there is any correlation between the presence of such lymphocytes and resistance to infection and our results suggest that this may be so. If this is confirmed it will become necessary to ascertain the effect of various vaccination procedures on the lymphocyte population as well as on the antibody titres. This may not be straightforward – we think that skin

sensitivity must arise as a result of exposure to natural infection and were therefore surprised not to be able to demonstrate its appearance after artificial or natural infection. Non-specific depression of cell-mediated immunity after influenza infection may well play a part (Reed, Olds & Kisch, 1972) but we think it is unlikely to be the whole explanation.

We would like to thank Mrs Carole Williams for technical assistance and the manufacturers for supplying valuable virus material.

REFERENCES

BEVERIDGE, W. I. B. (1952). Immunity to viruses – a general discussion with special reference to the role of allergy. *Lancet* ii, 303.

BEVERIDGE, W. I. B. & BURNET, F. M. (1944). A cutaneous reaction to the influenza viruses. *Medical Journal of Australia* i, 85.

DENMAN, E. J., DENMAN, A. M., GREENWOOD, B. M., GALL, D. & HEATH, R. B. (1970). Failure of cytotoxic drugs to suppress immune responses of patients with rheumatoid arthritis. *Annals of Rheumatic Diseases* **29**, 220.

FREESTONE, D. S., HAMILTON-SMITH S., SCHILD, G. C., BUCKLAND, R., CHINN, S. & TYRRELL, D. A. J. (1972). Antibody responses and resistance to challenge in volunteers vaccinated with live attenuated, detergent split and oil adjuvant A2/Hong Kong/68 (H_3N_2) influenza vaccines. *Journal of Hygiene* **70**, 531.

HOBSON, D., BEARE, A. S. & WARD GARDNER, A. (1971). In *Proceedings of a Symposium on Live Influenza Vaccine* (ed. B. Gušić), p. 73. Zagreb: Yugoslav Academy of Sciences and Arts.

REED, W. P., OLDS, J. W. & KISCH, A. L. (1972). Decreased skin-test reactivity associated with influenza. *Journal of Infectious Diseases* **125**, 398.

SCHILD, G. C., HENRY-AYMARD, M. & PEREIRA, H. G. (1972). A quantitative single-radial-diffusion test for immunological studies with influenza virus. *Journal of General Virology* **16**, 231.

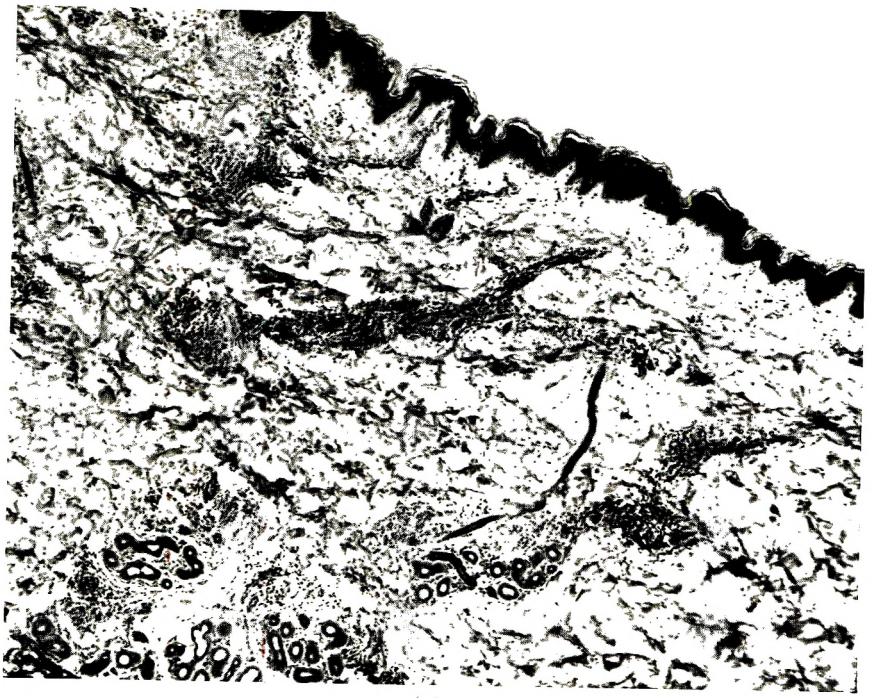
TYRRELL, D. A. J., PETO, M. & KING, N. (1967). Serological studies on infections by respiratory viruses of the inhabitants of Tristan da Cunha. *Journal of Hygiene* **65**, 327.

WALDMAN, R. H. & HENNEY, C. S. (1971). Cell-mediated immunity and antibody response in the respiratory tract after local and systemic immunization. *Journal of Experimental Medicine* **134**, 482.

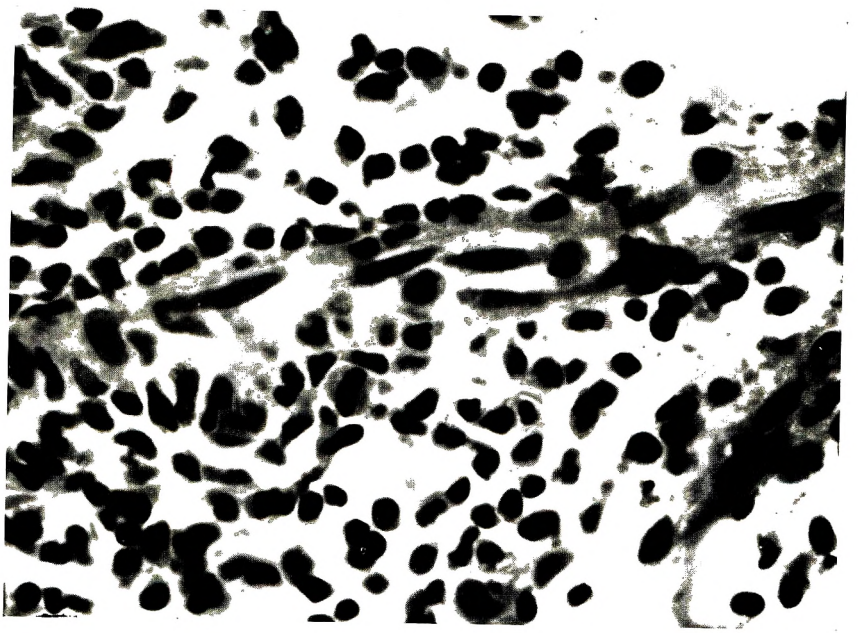
EXPLANATION OF PLATE

PLATE 1

(*a*) Biopsy from subject injected with influenza A material (stained with haematoxylin and eosin, × 32). Blood vessels show well-marked cuffing by lymphocytic and mononuclear infiltrate. A less-intense cell infiltration extends deeply into dermis in region of sweat glands. (*b*) Biopsy from subject injected with influenza B material (haematoxylin and eosin, × 440). Capillary is surrounded by infiltrate consisting largely of lymphocytes with occasional histiocytic type cells.



(*a*)



(*b*)

J. Hyg., Camb. (1973), **71**, 761
Printed in Great Britain

Studies on the microbial flora in the air of submarines and the nasopharyngeal flora of the crew

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(*Received* 30 *March* 1973)

SUMMARY

Airborne bacteria surveys in nuclear submarines show that the total microbial load was maintained at satisfactorily low levels during prolonged patrols despite factors which were expected to increase this form of pollution.

The isolation rates of *Staphylococcus aureus* and *Neisseria meningitidis* from nasal and nasopharyngeal swabs respectively, together with the serum antibody titres to *Mycoplasma pneumoniae*, before and after patrols suggested that the transmission of these organisms between individuals was not much increased by patrol conditions. The finding of higher numbers of airborne gram-negative rods and bodily contamination by enterobacteria is frequently reported in submersibles but does not appear to cause major outbreaks of illness in nuclear submarine crews.

INTRODUCTION

The introduction of nuclear submarines and space capsules in which men live in sealed environments brought many new problems in atmospheric control (Ebersole, 1960; Lambert, 1970), especially in situations where even minor illness can determine the success or failure of a mission.

The microbiological implications of confining men for several weeks in submarines with continuously recycled air systems were examined by Watkins *et al.* (1970) who emphasized the need for systemic surveillance and pre-patrol screening procedures. They showed that in addition to those factors normally associated with military populations in barracks and ships, many others affect the bacteria load in a submarine. Some components of the air revitalization system are bactericidal, though not specifically designed for this purpose. The catalytic burners used to remove carbon monoxide, for example, operate at around 500° F. and it is worthy of note that Bourdillon (1945) suggested heating air to this temperature as a method of destroying airborne micro-organisms in ships when respiratory tract infections caused concern during World War II.

Watkins and his colleagues found that a major factor influencing the concentration of airborne bacteria was the inboard venting of excess air from the sewage tanks following their discharge by high pressure air at depth. After venting, transitory counts of the order of 10^5 bacteria per cubic foot of air were found, and

organisms of faecal origin were recovered in a high proportion of throat and nasal swabs from the crews.

In view of these findings, a small working party was set up at the start of the British Polaris submarine programme to assess the risks of infection in submarine personnel, and if necessary to advise on measures to reduce them. Four main objectives were established, namely:

1. To assess the airborne microbial contamination of submarines during prolonged patrols.

2. To follow the spread of indicators of infection among the crews, for example, *Staphylococcus aureus* in nasal swabs, *Neisseria meningitidis* in nasopharyngeal swabs and serum antibody titres to infective agents such as *Mycoplasma pneumoniae*.

3. To provide medical officers accompanying the patrols with materials and directions for the collection, identification and preservation of organisms isolated during patrol for subsequent study in shore reference laboratories.

4. To analyse the reasons for sick bay attendance on patrols.

MATERIALS AND METHODS

Environmental studies

The Bourdillon (Bourdillon, Lidwell & Thomas, 1941) slit sampler was used to determine the number of airborne particles carrying viable bacteria: total counts were estimated from the resultant colonies on Oxoid blood agar base enriched with 5% horse blood or serum using a 2 min. (= 60 litres, $\simeq$ 2 cubic feet air) sample time. Counts of organisms of bowel origin were made on Oxoid MacConkey agar No. 2 using a 5 or 10 min. sampling time ($\simeq$ 5 or 10 cubic feet samples respectively).

Attempts were also made to estimate airborne concentrations of *Streptococcus salivarius* by the method of Willlams & Hirch (1950) and of *Staph. aureus* using various selective media (Harding & Williams, 1969) but these failed largely because of the difficulties of preparing or storing complex media on board the submarine.

After collection of the air samples, the resultant colonies were counted after 24 and 48 hr. incubation at 37° C. The results reported here were usually based on the 48 hr. count, but swarming colonies of *Proteus* or *Bacillus* species occasionally interfered and in these cases the 24 hr. counts were included. Attempts were made to identify the genera isolated with the limited range of simple tests available on board and random colonies were subcultured to maintenance media for subsequent identification in shore laboratories. The nomenclature and characterization tests of Cowan & Steel (1965) were used throughout.

Human studies

Nasal swabs for Staphylococcus aureus

Samples were collected on plain cotton-wool swabs, spread directly on salt mannitol agar plates and incubated at 37° C. for 18–24 hr. Presumptive *Staph. aureus* colonies were tested for coagulase production and positive strains phage

typed and tested for sensitivity to penicillin, streptomycin, chloramphenicol, tetracycline and erythromycin using Oxoid Multodisks.

Nasopharyngeal swabs for Neisseria meningitidis

On early patrols, nasopharyngeal swabs were spread directly on heated blood agar plates which, after incubation for 18–24 hr. at 37° C. in 10 % carbon dioxide, were examined for characteristic colonies of *N. meningitidis*, the identity of which was then confirmed by standard methods. A 20 % isolation rate of *N. meningitidis* in the first complete Polaris crew examined was followed by very low rates in succeeding surveys and these were thought to be due to collection and technical difficulties.

The method which yielded the highest recovery rate and most consistent results was that in which samples were collected on cotton wool swabs supported on applicators bent at an angle of 30°. They were spread directly on prewarmed plates of Difco Mueller-Hinton agar containing 300 μg. vancomycin, 750 μg. colistin and 1250 units nystatin per 100 ml. Plates were incubated at 37° C. in 10 % CO_2 for 18–24 hr. and colonies of *N. meningitidis* further identified by standard methods prior to testing for sulphonamide resistance and antigenic typing (R. J. Fallon and P. H. Marsden, unpublished).

Nasopharyngeal swabs for total and differential colony counts of bacteria

During the studies on meningococcal carrier rates, large numbers of gram-negative rods, chiefly of the Enterobacteriaceae but also many pseudomonads, were isolated from nasopharyngeal swabs. Semi-quantitative studies were therefore carried out to determine the extent, frequency and duration of throat carriage of these organisms. The technique used was that in which soluble alginate swabs on flexible wires were taken by one observer, well mixed in Calgon–Ringer solution and plate counts made by the technique of Miles & Misra (1938). All colonies were differentiated into Gram-positive cocci and rods and Gram-negative cocci and rods, and random colonies of Gram-negative rods from a proportion of the samples were identified by standard biochemical methods.

Serum antibody titres to Mycoplasma pneumoniae

Surveys for antibodies to *Mycoplasma pneumoniae* were carried out on two crews by complement fixation tests using the lipid antigen of *M. pneumoniae* (Kenny & Grayston, 1965) in the method of Bradstreet & Taylor (1962) as modified by Grist, Ross, Bell & Stott (1966) and by metabolic inhibition tests (Taylor-Robinson, Purcell, Wong & Chanock, 1966).

RESULTS

Airborne bacteria

The results summarized in Tables 1 to 5 were derived from surveys in three Polaris submarines during eight patrols of up to sixty days in which the effects of duration of patrol, time of day and occupational area on the number of viable

Table 1. *Effect of patrol time on the total airborne bacteria-carrying particle count. The mean colony count/ft.³ and standard error of the mean* (S.E.M.) *was derived from the indicated number of air samples after correction for the 2 ft.³ sample volumes*

	Number of air samples	Mean colony count/ft.³	S.E.M.
Before patrol	108	9·1	0·3
Weeks of patrol 1	102	11·0	0·4
2	48	15·5	0·9
3	88	16·0	0·8
4	76	16·2	0·9
5	46	15·8	0·8
6	—	—	—
7	56	15·9	0·9
8	30	15·8	1·2

Table 2. *Effect of patrol time on the number of airborne particles carrying organisms of bowel origin. The values were derived as for Table 1 except that correction was made for the 5 or 10 ft.³ sample volumes*

	Number of air samples	Mean colony count/ft.³	S.E.M.
Before patrol	40	0·52	0·03
Weeks of patrol 1	50	2·54	0·16
2	—	—	—
3	25	3·04	0·30
4	30	3·41	0·31
5	14	2·40	0·33
6	—	—	—
7	16	2·68	0·29
8	10	3·55	0·40

airborne bacteria are examined. There were no significant differences in the results between submarines or between the slit samplers used in these surveys.

There was a statistically significant increase in total airborne bacteria in the first week of patrol compared with the prepatrol period ($t = 3{\cdot}8$, $P < 0{\cdot}001$) and in the second patrol week compared with the first ($t = 5{\cdot}3$), $P < 0{\cdot}001$). There was no significant change during the remainder of patrol, the overall mean being 16 airborne bacteria-carrying particles per cubic foot ($\simeq 0{\cdot}45/m^3$) of air. The number of organisms of bowel origin was also significantly increased in the first week of patrol compared with prepatrol values ($t = 10{\cdot}1$, $P < 0{\cdot}001$) and remained higher throughout, but with more variations than in the total counts of airborne bacteria.

A steady increase in the number of airborne bacteria occurred during the working day and peak values were reached in the early evening when the majority of the crew were bathing, dining and taking part in social activities (Table 3). That higher counts were clearly associated with human activity is clear from Table 4 where the counts are very low in the relatively unoccupied working spaces.

The results of the post patrol identification studies are presented in Table 5 which summarizes the identity of some 3000 colonies subcultured to maintenance media during patrols. A little over 3% did not survive or were not identified.

Table 3. *Effect of time of day on the total airborne bacteria-carrying particle count. The values were derived as in Table* 1

	Number of air samples	Mean colony count/ft.^{3}	S.E.M.
Midnight – 04.00	46	8·5	0·3
04.00 – 08.00	50	9·0	0·3
08.00–12.00	128	12·5	0·5
12.00–16.00	116	14·3	0·8
16.00–20.00	126	19·4	1·1
20.00 – Midnight	88	11·5	0·5

Table 4. *Total airborne bacteria-carrying particle counts in different areas of the submarine. The values were derived as in Table* 1

	Number of air samples	Mean colony count/ft.^{3}	S.E.M.
Accommodation Spaces			
Galley	45	14·8	0·8
Bunk space	52	13·5	0·5
Messes	125	14·2	1·0
Heads and bathrooms	150	16·9	1·0
Average for overall accommodation spaces	372	15·3	—
Average for working spaces	90	5·2	0·15

Table 5. *Differential counts of airborne bacteria recovered from air samples during patrols*

Gram-positive species				Gram-negative species			
Spheres		Rods		Spheres		Rods	
Micrococcus	40·9 %	*Corynebacterium*	15·0 %	*Neisseria*	2·8 %	*Acinetobacter*	7·0 %
Staphylococcus	10·5 %	*Bacillus*	5·5 %			*Enterobacter*	6·5 %
Aerococcus	3·1 %					*Escherichia*	4·0 %
*Streptococcus**	2·9 %					*Proteus*	0·8 %
						Pseudomonas	0·6 %
						Alcaligenes	0·4 %
Total = 77·9 %				Total = 22·1 %			

* 62 % of these were *Strep. faecalis*.

Human studies

Staphylococcus aureus *in nasal swabs*

The nasal carriage rate of *Staph. aureus* and the proportion of strains resistant to the five antibiotics used routinely for testing are shown in Table 6. It is evident that although there were no significant changes in carriage rate during patrol, there was an apparent increase in the proportion of strains exhibiting antibiotic resistance.

There was no evidence of selection of particular phage types of *Staph. aureus* during patrols; there were 88 different phage types in 264 of the strains isolated before patrol with 27 strains untypable at 100 RTD and 56 different types in 150 strains isolated after patrol with 8 untypable.

Table 6. *The carriage rate and number of resistant strains of* Staph. aureus *in nasal swabs from submarine crews*

	Before patrol	During patrol	End of patrol
Number of swabs examined	917	175	493
Number positive for *Staph. aureus*	291 (31·7)*	58 (33·1)	158 (32·0)
Number resistant to			
Penicillin G	91 (31·3)	30 (51·7)	85 (53·8)
Streptomycin	4 (1·4)	4 (~ 7)	7 (4·4)
Tetracycline	4 (1·4)	4 (~ 7)	9 (5·7)
Erythromycin	0	0	4 (2·5)
Chloramphenicol	2 (0·7)	1 (~ 2)	2 (1·3)

* Figures in parentheses indicate percentages.

Table 7. *Nasopharyngeal carriage rates of* N. meningitidis *and the proportion of sulphadiazine resistant strains*

	Before patrol	End of patrol
Number of swabs examined	266	127
Number of swabs positive for *N. meningitidis*	71 (26·7)*	36 (28·3)
Number of strains growing in the presence of sulphadiazine (mg./100 ml. medium)		
1·0 (resistant)	0	2 (~ 5)
0·1 (partially resistant)	7 (~ 10)	7 (~ 19)
0·01 (sensitive)	70 (~ 84)	25 (~ 69)
Not tested	4	2

* Figures in parentheses indicate percentages.

Neisseria meningitidis *in nasopharyngeal swabs*

The isolation rates of *N. meningitidis* from nasopharyngeal swabs and the proportion of strains resistant or partially resistant to sulphadiazine are presented in Table 7. This work is incomplete and the figures are insufficient to allow firm conclusions to be drawn; they are shown here because the relatively unchanged isolation rates and suggestive increases in sulphadiazine resistant strains were similar to those observed with *Staph. aureus*. Further surveys are being carried out and the results will be reported later (P. H. Marsden & R. J. Fallon, unpublished.)

Streptococcus pyogenes *in throat swabs*

Only 13 isolations of Lancefield group A β haemolytic streptococci were made from some 1300 throat swabs examined before and after patrols.

Semi-quantitative estimates of nasopharyngeal flora

Table 8 summarizes the results of the semi quantitative assay of the nasopharyngeal flora of two crews before and after a patrol. The frequency distribution curve is not normally distributed, being skewed to the right, and a statistical treatment of the results by log probit analysis indicates that there is no significant change in the number of nasopharyngeal organisms as a result of patrol. There is, however, a clear-cut difference in the type of organisms recovered at the end of

Table 8. *Estimates of the number and type of nasopharyngeal organisms in submarine crews before and after a patrol. Values are based on the log of the probable number of organisms per swab from a Miles & Misra* (1938) *count, and the standard deviations* (S.D.) *by log probit analysis*

	Before patrol	End of patrol
Number of swabs examined	217	165
Viable organisms/swab $\times 10^3$ Log mean	22·5	26·5
Range (-2 S.D.)	16·0	18·8
($+2$ S.D.)	40·5	47·8
Gram-positive cocci (mainly *Staph.*, *Strep.* and *Micrococ.* spp.)	58 %	20 %
Gram-positive rods (*Bacillus*, *Corynebacterium* spp.)	12 %	24 %
Gram-negative cocci (*Neisseria* spp.)	10 %	10 %
Gram-negative rods (Mainly *Haemophilus* spp. and enterobacteria)	10 %	46 %

patrol compared with the beginning, with a fall in the proportion of gram-positive cocci and a corresponding rise in gram-negative rods. The types of gram-negative organisms isolated were similar to those found in air samples and include *Acinetobacter*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Proteus* and *Pseudomonas* species. *Strep. faecalis* was also found frequently.

Serum antibody titres to Mycoplasma pneumoniae

M. pneumoniae antibodies were found in 84/412 (20·4 %) of pre-patrol sera and in 27/140 (19·3 %) post-patrol sera tested by complement fixation; in 195 pre-patrol sera tested by both complement fixation and metabolic inhibition (neutralization) tests, antibodies were found in 40 (20·5 %) by MIT, 36 (18·5 %) by CFT and 63 (32·3 %) by one or the other test. These rates were similar to those for civilian sera tested in the same laboratory (R. J. Fallon, unpublished data).

DISCUSSION

On the evidence of slit sampler counts of total airborne bacteria-carrying particles, microbial pollution of the submarine atmosphere remains at an acceptably low figure during long patrols and the air revitalization system can easily cope with the increases associated with human activity. The values compare favourably with those quoted by Williams, Lidwell & Hirsch (1956) in schools, offices and factories and are significantly lower than those reported by Ellis & Raymond (1945) in the overcrowded ships of World War II. The results presented here are not strictly comparable with those of Watkins *et al.* (1970) for U.S. Navy Polaris submarines since the latter used raised jet impingers to collect 20 min air samples in 5 % skim milk saline which were then frozen until the end of patrol for analysis in shore laboratories. Impinger counts are usually higher than those derived by slit sampler methods, but some of the U.S. Navy figures appear even higher than would be explained by the difference in methods and the occasional very high counts, of the order of 3×10^4 organisms per cubic foot of air ($\simeq 10^3/\text{m.}^3$),

were not seen in the present study. The question arises whether multiplication of some organisms during storage and assay contributed to such high results.

The use of bacterial counts to indicate the hygienic state of the air in occupied spaces has been investigated for many years without a satisfactory conclusion being reached (Wilson & Miles, 1964; R. E. O. Williams, personal communication, 1968). Reid, Lidwell & Williams (1956) observed positive correlations between various respiratory infections in school children and general flora counts of their classrooms, but concluded that these relationships would not justify the use of total airborne bacteria counts as indices of the risk of infection. There is general agreement that in assessing the risk to the inhabitants of a closed space, it is not the total microbial load, but the number of airborne pathogens which is important. This number may be very small (Riley, 1957) and not detectable by normal sampling methods, it also varies for different species. Some workers have therefore used counts of 'indicator' organisms to assess the risks in the same way as the probable coliform count is used in water testing.

Table 2 shows that about one fifth of the airborne bacteria in a dived submarine are probably of bowel origin as indicated by MacConkey agar counts, and this is supported by the differentiation of organisms from the blood agar plates (Table 5).

The numbers and proportions of coliform organisms and enterococci are higher than in Williams's 1956 surveys but appear to be common in submerged vessels. Davies, Valentine & Feindler (1970), in detailed surveys of the water systems, human flora and general environment of the *Ben Franklin* submersible used as a space station analog, also observed widespread contamination with gram-negative rods, notably *Pseudomonas*, *Escherichia* and *Proteus* species.

There is general agreement that organisms of bowel origin can readily be recovered from the upper respiratory tract of the crews of these ships. In addition, the American workers found significant increases in enterobacterial contamination of the skin despite, in the case of the *Ben Franklin*, positive attempts to reduce it by antimicrobial soaps and treated garments. The shift to predominantly gram-negative organisms in the nasopharyngeal flora, reported in the *Ben Franklin*, is repeated in the British Polaris submarine crews. Whether these shifts are due to aerosols generated in the submarine or whether the ambient conditions (for example, raised carbon dioxide affecting tissue pH), favour survival of particular species is not yet clear, but it is likely that the former is the main factor. In repeated nasopharyngeal swabs from thirty individuals on patrol, for instance, the predominant organisms on one particular day were *Pseudomonas* species, but three days later *Strep. faecalis* was the most frequent isolate. Ten days later, *Proteus* and *Esch. coli* were recovered from the majority of the swabs whilst towards the end of patrol, *Pseudomonas* reappeared as the major component. It is of interest that on this patrol a minor outbreak of otitis externa was shown by pyocine typing to be due to the same strain of *Ps. aeruginosa* as that isolated from apparently healthy throats a few days earlier.

Survey of the spread of indicators of infection yielded largely negative, and therefore encouraging results. There was no increase in the nasal carriage rate of *Staph. aureus* and little or no evidence of selection of particular phage types of

these organisms during patrols. It was occasionally possible to follow an individual strain and in the two main cases where this occurred it may be significant that medical staff appeared to be the originators. A penicillin resistant strain of *Staph. aureus,* phage type 29/52/52A/53/54/79/80/85 isolated from a medical technician at the start of one patrol was subsequently cultured at the end of patrol from four of his shipmates and from a further seven by the end of the next patrol. A similarly well defined strain was transferred from the doctor to some of his patients in another crew.

The isolation rates of *Staph. aureus* in nasal swabs and *Neisseria meningitidis* in nasopharngeal swabs and the serum antibody titres to *Mycoplasma pneumoniae* suggest that the crew's experience of infection by these organisms is similar to that of the general population. In view of the infrequent use of antibiotics during the patrols studied, the finding of an apparent increase in the number of resistant strains of *Staph. aureus* is interesting, especially as a similar trend was seen with respect to *N. meningitidis* isolated from nasopharyngeal swabs.

Despite the apparent dangers due to the presence of organisms of bowel origin in air samples and throat and nasal swabs of the crews, there is little evidence of illness as a result. Few experiments appear to have been reported on respiratory tract infections by bowel pathogens although much work has been carried out on the transmission of aerosols carrying faecal organisms. Darlow & Bale (1959) and Newsom (1972) examined the microbial hazards associated with flushing lavatories but the submarine situation is not comparable because high pressure air is released from the sewage tank after thorough agitation of the contents. Darlow, Bale & Carter (1961) showed that the lethal dose of *Salmonella typhimurium* in mice was lower when they were infected by inhalation than by ingestion, but they were concerned with much larger doses of the infectious agent($\sim 5 \times 10^4$ orgs/dose) than slit sampler counts show to be possible in the air of RN submarines. On the other hand, if the highest impinger counts of Watkins *et al.* (1970) are correct, there is reason for concern.

A detailed report on the frequency of and reasons for sick bay attendance is being prepared for publication, but United States Navy and Royal Navy experience is that the sickness rate falls during patrols and is lower in each patrol than the preceding one by the same crew. There is a highly significant reduction in sick bay attendance for upper respiratory complaints after the first two weeks of patrol, and it is concluded that the presence of microbial contaminants at the levels observed do not represent a threat to the wellbeing of the crews.

This work was carried out with the permission of Flag Officer Submarines and we are indebted to the Commanding Officers, Medical Officers and medical staff of HM Submarines *Resolution*, *Revenge* and *Repulse* for their full hearted co-operation. We are grateful for the technical assistance of the staff of the Department of Laboratory Medicine, Ruchill Hospital and in particular to Mr W. M. Brown, FIMLT. Also the Phage-typing Laboratory, Department of Bacteriology and Immunology, Western Infirmary, Glasgow for typing the *strains* of *Staph. aureus* and Dr J. R. W. Govan, Edinburgh Royal Infirmary for pyocine typing of strains of *Ps. aeruginosa.* Acknowledgements are also due to the Medical Director

General (Navy) and the Royal Naval Personnel Research Committee of the Medical Research Council for permission to publish the findings.

REFERENCES

BOURDILLON, R. B. (1945). Memorandum to Air Hygiene Committee of the Royal Naval Personnel Research Committee, Medical Research Council.

BOURDILLON, R. B., LIDWELL, O. M. & THOMAS, J. C. (1941). A slit sampler for collecting and counting airborne bacteria. *Journal of Hygiene* **41**, 197.

BRADSTREET, C. M. P. & TAYLOR, C. E. D. (1962). Technique of complement-fixation test applicable to the diagnosis of viral diseases. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **21**, 96.

COWAN, S. T. & STEEL, K. J. (1965). *Manual for the Identification of Medical Bacteria.* Cambridge University Press.

DARLOW, H. M. & BALE, W. R. (1959). Infective hazards of water closets. *Lancet* i, 1196.

DARLOW, H. M., BALE, W. R. & CARTER, G. B. (1961). Infection of mice by the respiratory route with *Salmonella typhimurium*. *Journal of Hygiene* **59**, 303.

DAVIS, R. F., VALENTINE, O. & FEINDLER, K. (1970). *Use of the* Ben Franklin *as a Submersible Space Station Analog*, vol. IV, Grumman Aerospace Corporation, New York.

EBERSOLE, J. H. (1960). The new dimensions of submarine medicine. *New England Journal of Medicine* **262**, 599.

ELLIS, F. P. & RAYMOND, W. F. (1945). The bacterial content of the air of HM Ships under wartime conditions. *Royal Naval Personnel Research Committee Report* RNP.45/220, Medical Research Council.

GRIST, N. R., ROSS, C. A. C., BELL, E. J. & STOTT, E. J. (1966). *Diagnostic Methods in Clinical Virology.* Blackwell Scientific Publications, Oxford.

HARDING, L. & WILLIAMS, R. E. O. (1969). Selection of *Staphylococcus aureus* in cultures from air samples. *Journal of Hygiene* **67**, 35.

KENNY, G. E. & GRAYSTON, J. T. (1965). Eaton Pleuropneumonia-like Organism (*Mycoplasma pneumoniae*) complement-fixing antigen: extraction with organic solvents. *Journal of Immunology* **95**, 19.

LAMBERT, R. J. W. (1970). Preventive medicine in the nuclear submarine programme. *Journal of the Royal Naval Medical Service* **56**, 176.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *Journal of Hygiene* **38**, 732.

NEWSOM, S. W. B. (1972). Microbiology of hospital toilets. *Lancet* ii, 700.

REID, D. D., LIDWELL, O. M. & WILLIAMS, R. E. O. (1956). Counts of airborne bacteria as indices of air hygiene. *Journal of Hygiene* **54**, 524.

RILEY, R. L. (1957). Aerial dissemination of pulmonary tuberculosis. *American Review of Tuberculosis* **76**, 931.

TAYLOR-ROBINSON, D., PURCELL, R. H., WONG, D. C. & CHANOCK, R. M. (1966). A colour test for the measurement of antibody to certain mycoplasma species based on the inhibition of acid production. *Journal of Hygiene* **64**, 91.

WATKINS, H. M. S., MAZZARELLA, M. A., MEYERS, C. E., HRESKO, J. P., WOLOCHOW, H., CHATIGNY, M. A. & MORGAN, F. M. (1970). In: *Proceedings of the Third International Symposium on Aerobiology.* Ed. I. H. Silver, pp. 9–40. London: Academic Press.

WILLIAMS, R. E. O. & HIRCH, A. (1950). The detection of streptococci in air. *Journal of Hygiene* **48**, 504.

WILLIAMS, R. E. O., LIDWELL, O. M. & HIRCH, A. (1956). The bacterial flora of the air of occupied rooms. *Journal of Hygiene* **54**, 512.

WILSON, G. S. & MILES, A. A. (1964). In: *Topley and Wilson's Principles of Bacteriology and Immunity*, 5th Edition, pp. 2483–2496. London: Edward Arnold.

J. Hyg., Camb. (1973), **71**, 771
Printed in Great Britain

The incidence of antibiotic resistance and other characteristics amongst *Escherichia coli* strains causing fatal infection in chickens: the utilization of these characteristics to study the epidemiology of the infection

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(*Received* 2 *April* 1973)

SUMMARY

Of 173 epidemiologically unrelated strains of *Escherichia coli* isolated from the pericardial sac of chickens that had died from infection with these organisms in England in 1972, approximately 1 year after the introduction of legislation forbidding the routine use of feeds containing 'therapeutic' antibiotics, 83·8% were resistant to sulphonamides, 31·2% to tetracyclines, 20·8% to furazolidone, 18·5% to streptomycin, 2·9% to spectinomycin and 1·2% to ampicillin; none of the strains were resistant to chloramphenicol, neomycin, polymixin, trimethoprim or nalidixic acid. The sulphonamide resistance and possibly some of the resistance to other agents might have been the consequence of sulphonamides being exempted from the legislation. Much of the resistance, with the exception of that to furazolidone, was of the transferable type. Many strains possessed transfer factors in the absence of any known transferable characteristic. Colicine production was twice as common in the pathogenic strains as in a collection of strains isolated from the faeces of healthy chickens; about half of it was transferable.

By means of serology, antibiotic resistance and other markers, it was found that several different kinds of *E. coli* were usually incriminated in any one outbreak of *E. coli* infection in broiler chickens. Sometimes the same kinds of *E. coli* were found in outbreaks in consecutive crops of chickens on the same farm. New kinds, too, appeared to be brought in by replacement chickens.

INTRODUCTION

The routine use of tetracyclines as feed additives has given rise to large populations of tetracycline-resistant *Escherichia coli* in the alimentary tract of chickens (Smith & Crabb, 1957). There is little doubt that it has also been responsible, in great part, for the high incidence of tetracycline resistance found amongst strains of *E. coli* pathogenic for poultry (Sojka & Carnaghan, 1961). Following the report of the Swann Committee (Report, 1969), legislation was introduced in Britain in March 1971 prohibiting the use of 'therapeutic' antibiotics, except sulphonamides, as feed additives; their use for disease control on veterinary prescription, however,

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continued. It seemed worth while therefore to study the antibiotic resistance of strains of *E. coli* that had killed broiler chickens in Britain in 1972 to determine whether this legislation had had a more immediate effect and to form a 'base-line' for future assessments of its long-term effect. The results are presented here. The strains were also examined for antibiotic resistance, R factors, non-transmissible but mobilizable resistance determinants, transfer factors, other transferable characters such as colicine (Col) and haemolysin (Hly) production and for lysogenicity. The information gained was then used to augment serological typing in studying the epidemiology of *E. coli* infection, because the value of the latter is limited by the fact that the majority of pathogenic poultry strains belong to one or other of only three serotypes, O2:K1, O1:K1 and O78:K80 (Sojka & Carnaghan, 1961; Harry, 1964).

MATERIALS AND METHODS

Escherichia coli *strains*

These were obtained by culturing on MacConkey's agar the diseased pericardial sac of broiler chickens which, at autopsy, exhibited pathological lesions typical of *E. coli* infection. From the heavy *E. coli* growth that resulted, a single colony was picked, purified by replating and finally cultured on Dorset's egg medium. This was then maintained at 5° C. until required.

Escherichia coli *strains from healthy chickens*

These were obtained from the faeces of chickens kept on different farms; they were not submitted for serotyping.

Serological examination

Glucose agar cultures were submitted to slide agglutination tests with O78:K80 and O2:K1 antisera. Those that agglutinated with the former antiserum were accepted as O78:K80. Those that agglutinated with the latter were made into a thick suspension with nutrient broth and autoclaved at 121° C. for 30 min. to destroy the K antigens and expose the O antigens. The suspensions were then centrifuged, resuspended in broth and submitted to slide agglutination tests employing O1:K1 and O2:K1 antisera suitably diluted to enable O suspensions of known O1:K1 and O2:K1 strains to be differentiated from each other.

Antibiotic-sensitivity tests

These were performed by the disk method described by Smith (1970) using an Oxoid Multodisk (1744E) composed of eight disks containing (i) streptomycin (Sm) 25 μg., (ii) ampicillin (A) 25 μg., (iii) oxytetracycline (T) 50 μg., (iv) chloramphenicol (Cm) 50 μg., (v) neomycin (Neo) 30 μg., (vi) nalidixic acid (Nal) 30 μg., (vii) furazolidone (F) 15 μg. and (viii) sulphonamides (Su) 300 μg., and three separate disks containing spectinomycin (Sp) 25 μg., polymixin (Pol) 300 units and sulphamethoxazole/tri-methoprim (SXT) 23·75 μg. and 1·25 μg. respectively. No difficulty was experienced in making readings; the zones of bacterial inhibition surrounding the disks were either wide, very narrow or absent.

Transfer of antibiotic resistance in vitro

This was performed by the method described by Smith (1970), the prospective recipient strain being a nalidixic acid-resistant mutant (*nal*^{r}) of *E. coli* K 12 that was non-lactose fermenting (*lac*$^-$). The latter property provided an additional method of distinguishing the recipient strain from the prospective donor strains which, apart from being *nal*^{s}, were *lac*$^+$. The concentrations of antibiotics employed in the selection media were as described above except that furazolidone was used at 10 μg/ml. Strains that did not transmit their resistance were retested several times, with in addition a mutant of the K 12 recipient strain resistant to several different colicines; this was done because most of the prospective donor strains examined were colicinogenic for the usual recipient strain and sometimes, in mixed culture, had a marked depressant effect on its growth rate.

The identification of strains possessing only transfer factors or only resistance determinants

For this we used the mobilization test of Anderson (1965), the actual technique employed being that described by Smith & Linggood (1970). An *E. coli* K 12 strain possessing mobilizable determinants for streptomycin (Sm$^+$) and sulphonamide (Su$^+$) resistance was employed to identify strains possessing only transfer factors and K 12 strains possessing transfer factor F or I were used to identify strains possessing only mobilizable determinants.

Haemolysin and colicine production: their transferable nature

Haemolysin production was detected by spot-inoculating broth cultures on plates of 'washed blood' agar (Smith & Halls, 1967) and colicine production by spot-inoculation on plates of tryptose nutrient agar previously evenly spread with a broth culture of *E. coli* K 12 suitably diluted to produce just confluent bacterial growth after incubation. All plates were incubated at 37° C. for 24 hr. and read. The methods of Smith & Gyles (1970) were employed to determine whether or not the haemolysin and colicine production of individual strains were transferable, the indicator for colicine production being the *nal*^{r} *lac*$^-$ K 12 strain.

Colicine typing

The technique employed was that described by Lewis (1968), using indicator strains received from Professor P. Fredericq.

Identification of lysogenic strains

A broth culture of the *nal*^{r} *lac*$^-$ K 12 strain resistant to several different colicines was spread evenly over the surface of a plate of tryptose agar containing 0·02 M-$CaCl_2$ and 20 μg./ml. of sodium nalidixate. When dry, it was 'spot-inoculated' with young broth cultures of the strains of *E. coli* under test. The plates were incubated at 37° C. and examined for evidence of phage action.

RESULTS

Incidence of antibiotic resistance amongst strains of Escherichia coli *isolated from diseased chickens*

The antibiotic resistance found amongst 173 strains of *E. coli* isolated from the pericardial sac of chickens which had died in different outbreaks of clinical *E. coli* infection in England are illustrated in Table 1; over 60% of the strains belonged either to serotype O78:K80 or O2:K1. Only 13·3% of the strains were sensitive to all the 11 antibiotics against which they were tested. Sulphonamide resistance was by far the most common, followed by tetracycline, furazolidone and streptomycin resistance. Resistance to tetracyclines and streptomycin was found less frequently amongst the O2:K1 strains than amongst the others; the converse was true in the case of furazolidone resistance. With the exception of a few strains that were resistant to spectinomycin or ampicillin, all 173 were fully susceptible to the other seven antibiotics, including chloramphenicol, against which they were tested.

Sixteen different patterns of resistance were shown by the 173 strains. Apart from the large group that were resistant to sulphonamides only, none of the groups comprised 14% of the whole; most of them were much smaller. The resistance patterns were usually small, resistance to more than three antibiotics being uncommon.

The incidence of colicine and haemolysin production and lysogenicity amongst chicken strains of Escherichia coli

The results of examining the 173 pathogenic strains of *Escherichia coli* and 168 strains isolated from the faeces of healthy chickens from different farms for colicine production are summarized in Table 2. Most of the O78:K80 and O2:K1 strains and the other pathogenic strains produced colicines, the incidence being almost twice as high in these strains as in those isolated from the faeces of healthy chickens. Difficulty was experienced in identifying the actual colicines produced by the pathogenic strains as many strains produced more than one colicine. However, it was possible to divide all those that were identified into seven fairly well defined groups (1–7) according to the particular indicator strains which were susceptible to their colicinogenic activity. The percentage of the 173 epidemiologically unrelated pathogenic strains that fell into each group was: group 1, 28·5%; group 2, 20·9%; group 3, 7·6%; group 4, 13·9%; group 5, 5·1%; group 6, 13·3%; and group 7, 10·1%.

Only one of the 173 pathogenic strains and one of the 168 strains isolated from the faeces of healthy chickens were haemolytic. Twelve (6·9%) of the 173 pathogenic strains were lysogenic for *E. coli* K 12. Three belonged to serotype O78:K80 and one to serotype O2:K1.

Antibiotic resistance, colicine and haemolysin production; their transferable nature in the chicken strains of Escherichia coli

The incidence of transferable antibiotic resistance, colicine and haemolysin production in the 173 pathogenic strains of *E. coli* and in the 168 other strains is

Table 1. *Incidence of antibiotic resistance amongst strains of* Escherichia coli *isolated from diseased chickens*

Strains	Serotypes			
	O78:K80	O2:K1*	Other serotypes	All
No. examined . . .	43	63	67	173
% resistant to one or more drugs	83·7	88·9	85·1	86·7
% resistant to				
Sulphonamides	83·7	87·3	80·6	83·8
Tetracyclines	37·2	14·3	43·2	31·2
Furazolidone	18·6	28·6	14·9	20·8
Streptomycin	28·0	3·2	23·9	18·5
Spectinomycin	9·3	1·6	0	2·9
Ampicillin	0	1·6	1·5	1·2
Neomycin	0	0	0	0
Chloramphenicol	0	0	0	0
Nalidixic acid	0	0	0	0
Sulphamethoxazole/trimethoprim	0	0	0	0
Polymixin	0	0	0	0
% with resistance pattern†				
Su	30·0	44·5	35·9	37·6
Su T	18·6	9·5	15·0	13·9
Su F	2·3	22·2	1·5	9·3
Sm Su T	4·7	1·6	15·0	7·5
Sm Su T F	7·0	0	6·0	4·1
Su T F	4·7	3·2	4·5	4·1
Sm Su	4·7	3·2	0	2·3
Sm Su Sp F	2·3	1·6	0	1·2
Sm Su F	2·3	0	1·5	1·2
Sm Su Sp	4·7	0	0	1·2
F	0	1·6	1·5	1·2
A Sm Su T	0	0	1·5	0·6
Sm Su T Sp	2·3	0	0	0·6
A Su	0	1·6	0	0·6
Sm	0	0	1·5	0·6
T	0	0	1·5	0·6

* Includes two O1:K1 strains.

† A = ampicillin; F = furazolidone; Sm = streptomycin, Sp = spectinomycin; Su = sulphonamides; T = tetracyclines.

illustrated in Table 3. So is the incidence of transfer factors in those strains not shown to possess transferable characters. Most of the spectinomycin and ampicillin resistance and about half the tetracycline and streptomycin resistance was transferred to *E. coli* K12, the incidence of transferable streptomycin resistance being higher in the pathogenic group than in the other group. The incidence of transferable tetracycline resistance was lowest in the untyped pathogenic strains. Less than 20% of the sulphonamide resistance and none of the furazolidone resistance was transferable. Colicine production was transferable in about half the strains possessing this character, the incidence of transferability being similar in both groups. The incidence of transfer factors in strains not possessing known transferable characters was highest in the O78:K80 strains and in the untyped pathogenic strains. There

Table 2. *The incidence of colicine production amongst strains of* Escherichia coli *isolated from chickens*

	Source of strains				
	Diseased chickens				Healthy chickens
	O78:K80	O2:K1*	Other serotypes	Total	Untyped serologically
No. examined	43	63	67	173	168
No. colicine-positive	38	59	55	152	78
Colicine-positive (%)	88·4	93·6	82·1	87·8	46·4

* Includes two O1:K1 strains.

Table 3. *The incidence of transferable antibiotic resistance, colicine and haemolysin production and transfer factors in chicken strains of* Escherichia coli

	Source of strains				
	Diseased chickens				Healthy chickens
Characteristic*	O78:K80	O2:K1	Other serotypes	Total	Untyped serologically
Sm	9/12† (75·0)	0/2 (0)	7/17 (41·2)	16/31 (51·6)	7/27 (25·9)
Su	10/36 (27·8)	3/56 (5·4)	11/53 (20·8)	24/145 (16·5)	19/102 (18·6)
T	10/14 (71·4)	8/9 (88·9)	7/28 (25·0)	25/51 (49·0)	28/52 (53·8)
Sp	3/4 (75·0)	1/1 (100)	0/0	4/5 (80)	0/3 (0)
A	0/0	1/1 (100)	1/1 (100)	2/2 (100)	3/3 (100)
F	0/8 (0)	0/18 (0)	0/10 (0)	0/36 (0)	0/8 (0)
Col	22/38 (57·8)	30/59 (50·8)	30/55 (54·5)	82/152 (53·9)	41/78 (42·6)
Hly	0/0	0/0	0/1 (0)	0/1 (0)	0/1 (0)
Tra‡ only	6/12 (50·0)	4/21 (19·1)	15/25 (60·0)	25/58 (43·1)	16/90 (17·8)

The figures in parentheses are percentages.

* Sm, Su, T, Sp, A, F, Col, Hly and Tra are genetic determinants for streptomycin, sulphonamides, tetracyclines, spectinomycin, ampicillin and furazolidone resistance, colicine and haemolysin production and transfer factor.

† No. of strains in which it was transferable/no. of strains tested.

‡ Presence of transfer factor in absence of any characters shown to be transmissible.

were several examples of strains possessing transfer factors yet being unable to transfer their own tetracycline, streptomycin and sulphonamide resistance and their colicine production. By means of transfer factors F and I, it was possible to mobilize the non-transfering resistance possessed by three strains.

The isolation of different types of Escherichia coli *from outbreaks of* Escherichia coli *infection in broiler chickens*

The results of examining strains of *E. coli* isolated from the hearts of chickens that had died from *E. coli* infection on three broiler farms are summarized in Tables 4–6; the chickens belonged to one or other of three successive crops. Table 6 also shows the results for strains isolated from the infected yolk sacs of 20 one-day-old chicks belonging to the second crop of farm C which had died immediately before they were taken to the farm. None of the chickens on the farms had been given antibiotics or any other agents active against *E. coli*. Each farm consisted of 10–17 broiler houses sited close together and, although one particular kind of *E. coli* was sometimes isolated more commonly from dead chickens from one house than from another, this occurrence did not seem sufficiently common to justify considering each house as a separate unit.

Strains belonging to serotype O2:K1, O78:K80 and to other, unidentified, serotypes were present amongst those isolated from chickens from all three crops on all three farms. According to antibiotic sensitivity, production of colicine and haemolysin and lysogenicity, these could be further classified into a comparatively large number of different kinds, many of which were incriminated in the deaths that occurred in each crop, e.g. the number of kinds isolated from the dead chickens in the first crops examined from farms A, B and C were 10, 9 and 4 respectively. It was not unusual for the same kinds of *E. coli* to be isolated from some of the chickens in all three crops on each farm. A notable example of this was the finding of an antibiotic-sensitive, colicine-producing strain of O78:K80 *E. coli*, to be the most common kind causing disease in all three crops on farm B. A kind of *E. coli* O78:K80, sulphonamide and tetracycline-resistant, colicine-producing, and also lysogenic, was responsible for most of the deaths in the first and second crop on farm C; it was also isolated from dead chickens of the third crop but most of the deaths in this crop were caused by an O2:K1 kind of *E. coli*. These two kinds of *E. coli* O78:K80 were not commonly found amongst the epidemiologically unrelated strains illustrated in Table 1. Several kinds of *E. coli* isolated from chickens in the second and third crops of farm C resembled some of those that had been isolated from the infected yolk sacs of the dead chicks that would have formed part of the second crop. The colicine produced by the O2:K1 strain isolated from one of these chicks was the same as that produced by some of the O2:K1 strains that were isolated later from dead chickens of this crop and of the third crop.

A total of 198 strains of *E. coli* isolated from the hearts of chickens, one per chicken, that had died from *E. coli* infection on 36 other farms, 4–10 (average 5·5) per farm, were also examined. The number of kinds of *E. coli* amongst the strains from each farm varied from 1 to 6 (average 2·5); those from each of ten of the farms were of the same kind.

Table 4. *The different kinds of* Escherichia coli *isolated from 236 dead chickens in successive broiler crops on farm A*

Kind of *E. coli**	No. of strains isolated from dead chickens in crop no. 1	2	3
O2:K1			
Su Col	2	14	4
Su T Col	0	0	4
Su F Col	0	1	0
O78:K80			
Col	22	4	9
S Su Sp Col	34	4	0
Untyped†			
Col	4	8	0
Su	6	1	2
Su Col	15	39	0
Su T	0	7	0
Su T Col	8	7	8
Su T F Col	1	5	0
Su F Col	0	5	0
S Su T Col	3	5	10
S Su T F Col	1	3	0
All kinds	96	103	37

One strain from the heart of each dead chicken was examined.

* S, Sp, Su, T and F = resistant to streptomycin, spectinomycin, sulphonamides, tetracycline and furazolidone. Col = colicine-producing.

† Neither O78:K80, O1:K1 or O2:K1.

DISCUSSION

Despite the introduction of legislation, some 12 months previously, banning the use of 'therapeutic' agents as feed additives, the incidence of tetracycline and furazolidone resistance found in the present survey amongst strains of *Escherichia coli* pathogenic for poultry was still 31·2 and 20·8% respectively. It will be of interest to see whether or not these figures will decrease significantly with time, now that the use of these two agents in feeds has been restricted. The high incidence of resistance to sulphonamides, 83·8%, was not unexpected because it is still permissible to feed diets containing these agents continuously to poultry to prevent coccidiosis. It is doubtful whether the streptomycin resistance possessed by 18·5% of the pathogenic strains can be attributed to the selection pressure of streptomycin itself because this antibiotic is rarely given to poultry. It may well be a reflexion of the use of sulphonamides, as the genetic determinants for resistance to streptomycin and sulphonamides are often located on the same plasmid. It is possible, too, that the use of sulphonamides may be playing a part in maintaining the high incidence of tetracycline resistance because practically all the tetracycline-resistant *E. coli* examined were also resistant to these agents. If sulphonamides are acting in this manner it calls into question the advisability of

Table 5. *The different kinds of* Escherichia coli *isolated from 274 dead chickens in successive crops on farm B*

Kind of *E. coli**	No. of strains isolated from dead chickens in crop no. 1	2	3
O2:K1			
Su	0	0	7
Su Col	1	30	4
Su T Col	0	3	0
O78:K80			
Su	1	0	0
Col	38	42	24
S Su T Col	0	2	1
Untyped			
Col	1	0	0
Su Col	7	15	2
T	1	0	0
Su T Col	2	6	3
Su T F Col	0	3	0
S Su T	0	4	0
Su F Col	0	5	0
S Su T Col	2	29	5
S Su T F Col	19	0	0
Su Col Hly	0	15	2
All kinds	72	154	48

* Hly = haemolytic. For other details see Table 4.

Table 6. *The different kinds of* Escherichia coli *isolated from 200 dead chickens in successive broiler crops on farm C*

Kind of *E. coli**	No. of strains of this kind isolated from dead chickens in crop no. 1	2A†	2B	3
O2:K1				
Su Col	0	1	2	46
Su F Col	3	0	0	0
O78:K80 Su T Col Lys	73	0	10	12
Untyped				
Unclassifiable	0	1	2	1
Col	0	3	0	9
Su	1	4	1	1
Su Col	0	7	1	7
Su T Col	0	1	2	6
Su F Col	2	3	0	0
S Su T Col	0	0	0	1
All kinds	79	20	18	83

* Lys = lysogenic for *E. coli* K 12. For other details see Table 4.

† Strains from infected yolk sacs of 1-day-old chicks of crop no. 2; they had died immediately before being brought to farm C.

their continued use as feed additives unless, of course, it can be shown, as alleged, that they are essential in coccidiosis control. The complete absence of strains resistant to neomycin, chloramphenicol, nalidixic acid, trimethoprim and polymixin is in keeping with the fact that these agents are seldom, if ever, administered to poultry.

The failure to transfer furazolidone resistance from all 44 strains of *E. coli* examined confirms the observations of Smith (1966), from which it appears that transferable furazolidone resistance in *E. coli*, in contrast to resistance to most other antibiotics, must be uncommon. The high incidence of pathogenic strains, other than those of serotype O2:K1, possessing transfer factors and no identifiable transferable characteristics suggests that these strains, in fact, may possess transferable characteristics that, in some way, are associated with pathogenicity, as was found in the case of strains enteropathogenic for pigs (Smith & Linggood, 1970). It is noteworthy, too, that colicinogenicity, shown to be transferable in over 50% of the strains examined, was twice as common in the pathogenic strains as in those isolated from the faeces of healthy chickens.

The results of classifying strains from different outbreaks according to serology, antibiotic resistance pattern, colicine and haemolysin production and lysogenicity have served to augment those previously made by Harry (1964), Harry & Hemsley (1965) and Hemsley & Harry (1965) using serological methods only. They show, for example, that several different strains may be involved in any outbreak. This finding stresses the desirability of performing antibiotic sensitivity tests on a number of strains before selecting an antibiotic with which to treat a particular outbreak. It suggests, too, that vaccines giving coverage against a wide variety of strains would be essential if immunological procedures were to be successful in preventing such outbreaks. It is also apparent from the examination of strains from farms A, B and C that (1) pathogenic strains may persist in broiler houses and thus give rise to outbreaks of clinical infection in consecutive crops of birds and (2) new pathogenic strains can be brought in by the replacement chicks themselves.

We are grateful to Mr Philip Green for his capable technical help. Our thanks are also due to Professor P. Fredericq, Dr R. F. Gordon, Dr E. G. Harry, Mrs Anne Millington and Mr W. J. Sojka for assistance in various ways.

REFERENCES

Anderson, E. S. (1965). A rapid screening test for transfer factor in drug sensitive Enterobacteriaceae. *Nature, London* **208**, 1016–17.

Harry, E. G. (1964). A study of 119 outbreaks of coli septicaemia in broiler flocks. *Veterinary Record* **76**, 443–8.

Harry, E. G. & Hemsley, L. A. (1965). The relationship between environmental contamination with septicaemia strains of *Escherichia coli* and their incidence in chickens. *Veterinary Record* **77**, 241–5.

Hemsley, L. A. & Harry, E. G. (1965). Coliform pericarditis (colisepticaemia) in broiler chickens: a three year study on one farm. *Veterinary Record* **77**, 103–7.

Lewis, M. J. (1968). Transferable drug resistance and other transferable agents in strains of *Escherichia coli* from two human populations. *Lancet* i, 1389–93.

Report (1969). *Report of the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine*. London: H.M.S.O.

SMITH, H. W. (1966). The incidence of infective drug resistance in strains of *Escherichia coli* isolated from diseased human beings and domestic animals. *Journal of Hygiene* **64**, 465–74.

SMITH, H. W. (1970). The transfer of antibiotic resistance between strains of enterobacteria in chickens, calves and pigs. *Journal of Medical Microbiology* **3**, 165–80.

SMITH, H. W. & CRABB, W. E. (1957). The effect of the continuous administration of diet containing low levels of tetracycline on the incidence of drug resistant *Bacterium coli* in the faeces of pigs and chickens: the sensitivity of the *Bact. coli* to other chemotherapeutic agents. *Veterinary Record* **69**, 24–30.

SMITH, H. W. & GYLES, C. L. (1970). The relationship between different transmissible plasmids introduced by F into the same strain of *Escherichia coli* K 12. *Journal of General Microbiology* **62**, 277–85.

SMITH, H. W. & HALLS, S. (1967). The transmissible nature of the genetic factor of *Escherichia coli* that controls haemolysin production. *Journal of General Microbiology* **47**, 153–61.

SMITH, H. W. & LINGGOOD, M. A. (1970). Transfer factors in *Escherichia coli* with particular regard to their incidence in enteropathogenic strains. *Journal of General Microbiology* **62**, 287–99.

SOJKA, W. J. & CARNAGHAN, R. B. A. (1961). *Escherichia coli* infection in poultry. *Research in Veterinary Science* **2**, 340–52.

J. Hyg., Camb. (1973), **71**, 783
Printed in Great Britain

A single medium for the rapid detection of *Escherichia coli* at 44° C

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(*Received* 6 *April* 1973)

SUMMARY

Lactose tryptone ricinoleate broth (0·3 % lactose, 2 % Oxoid tryptone, 0·1 % Na ricinoleate; pH 7·1) was found to be as good as the currently recommended combination of lactose ricinoleate broth and tryptone water for the detection of *Escherichia coli* in the positive tubes of presumptive coliform counts.

INTRODUCTION

British practice (Report, 1969) for the rapid detection of *Escherichia coli* in positive tubes of presumptive coliform counts requires that a tube of lactose ricinoleate broth (LRB) containing 1 % lactose and a tube of tryptone water (TW) should be inoculated with a loopful of culture from the positive presumptive tube; *E. coli* is judged to be present if gas is produced in LRB and indole in TW after 24 hr. at 44° C. The work involved in media preparation and subculturing and the requirement for water bath space would be halved if there were a single medium for the detection of lactose fermentation and indole production. The concentration of lactose in such a medium is important since Boyd & Lichstein (1965) found that the tryptophanase activity of *E. coli* was inhibited in media containing more than 0·02 M carbohydrate. This note describes a low lactose, tryptone rich ricinoleate medium suitable for the detection of both gas and indole by *E. coli* after incubation at 44° C for 20–24 hr.

MATERIALS AND METHODS

Samples of sewage effluent from Kariobangi sewage treatment works, Nairobi, of polluted water from various streams in Nairobi and of upland catchment water from Ruiru Dam, Nairobi, were used to inoculate suitable volumes of Oxoid MacConkey broth (purple). Some samples of polluted stream water were subjected to the chlorination treatment described by Burman (1967); others were stored at 4–6° C. for 14 days. These treatments were done to obtain physiologically debilitated organisms in an attempt to create conditions under which false reactions (either positive or negative) might occur. After these had been incubated for 14–48 hr. at 37° C., loopfuls of each resulting culture were transferred to tubes of LRB, TW and lactose tryptone ricinoleate broth (LTRB). LTRB contained 0·3 %

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Table 1. *Experimental details and results*

Sample source	No. of presumptive coliform cultures obtained	No. of cultures containing *E. coli* based on	
		LRB + TW	LTRB
Sewage effluent	50	50	50
Ruiru dam	25	0*	1*
Polluted waters:			
(i) untreated	60	60	60
(ii) chlorinated	20	20	20
(iii) stored at 4–6° C	25	7*†	8*†

* Disparity explained in text.
† Only one disparity.

lactose, 2 % Oxoid tryptone and 0·1 % sodium ricinoleate (pH 7·1); it was sterilized at 115° C. for 20 min. After incubation for 20–24 hr. at 44° C., the tubes were examined for gas and indole production, Kovac's reagent (Report, 1969) being used for the latter. Immediately before the addition of Kovac's reagent, plates of Oxoid eosin methylene blue (EMB) agar (Levine) were inoculated with the LTRB cultures; these were incubated at 37° C.

RESULTS AND DISCUSSION

One hundred and eighty coliform cultures were obtained: their sources and the number of cultures containing *E. coli* (as judged by both LRB + TW and LTRB) are given in Table 1. In only two cases was there a difference between the two methods. In each of these cases it was found to be caused by a false negative reaction in LRB + TW (no indole production in TW; however, when the positive LRB cultures were transferred into fresh TW, indole production at 44° C occurred). All the positive LTRB cultures formed green colonies with a metallic sheen, typical of *E. coli*, on EMB agar; none of the negative cultures did.

Gas production in LTRB was at least as copious as that in LRB. This suggests that the formulation of LRB with 1 % lactose is unnecessarily extravagant. It may be noted that American media for the presumptive and completed tests are formulated with only 0·5 % lactose (APHA, 1971). The medium for the confirmed test, however, is formulated with 1 % lactose.

The present results indicate that LTRB is at least as good as LRB + TW in confirming the presence of *E. coli* in presumptive positive tubes. Its greater convenience suggests that it is a practical alternative to the presently recommended procedure.

I am grateful to Oxoid Ltd. who kindly supplied a sample of Na ricinoleate.

REFERENCES

APHA (1971). *Standard Methods for the Examination of Water and Waste water*, 13th ed. New York: American Public Health Association.

Boyd, W. L. & Lichstein, H. C. (1955). The effect of carbohydrates on the tryptophanase activity of bacteria. *Journal of Bacteriology* **69**, 584–9.

Burman, N. P. (1967). Recent advances in the bacteriological examination of water. In: *Progress in Microbiological Techniques* (ed. C. H. Collins), pp. 185–212. London: Butterworths.

Report (1969). The bacteriological examination of water supplies. *Reports on Public Health and Medical Subjects No.* 71, 4th ed. London: H.M.S.O.

J. Hyg., Camb. (1973), **71**, 787
Printed in Great Britain

Dispersal and transfer of *Staphylococcus aureus* in an isolation ward for burned patients

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(*Received* 9 *April* 1973)

SUMMARY

The dispersal of *Staphylococcus aureus* from burned patients, the relation between nasal carriage by the staff and exposure to airborne *Staph. aureus*, and the transfer of *Staph. aureus*-carrying particles within the ward have been studied. The dispersal of *Staph. aureus* from burned patients was correlated to the size of the burn wound. The median values varied from 21 c.f.u./m.^{2}/hr. for patients with small burns to 453 c.f.u./m.^{2}/hr. for extensively burned patients. The size of the dispersed particles appeared to be smaller than that usually found in hospital wards. Carriage of nasal strains by the staff was correlated to the air counts; the number of patient sources did not seem to be of great importance. The transfer of *Staph. aureus* within the ward was at least 6 to 20 times that which would have been expected if transfer was due to air movement only.

INTRODUCTION

In a previous epidemiological investigation from an isolation ward for burned patients it was shown that in spite of the design of the ward there was a considerable amount of cross-infection with *Staphylococcus aureus* (Hambraeus, 1973).

In order to estimate the role of airborne transfer of infection, studies with an airborne particle tracer were carried out in the ward. According to this investigation, the airborne transfer of particles from room to room was small (Hambraeus & Sanderson, 1972). However, as burned patients are sometimes heavy dispersers of bacteria, even a small amount of airborne room-to-room transfer might give high air counts in the receiving rooms. The size of the bacteria-carrying particles is another factor that influences the airborne room-to-room transport of bacteria. In the earlier particle tracer investigation a particle with a sedimentation rate of 0·3 m./min. was used, as this is the median value found for airborne particles carrying *Staph. aureus* in hospital wards (Noble, Lidwell & Kingston, 1963). The median sedimentation rate for *Staph. aureus*-carrying particles in a burns ward might differ from this. In addition to transfer of staphylococci from one room to another by air movement, there might also be a contribution to the air counts in the room by carriers among the staff. Another possibility is transport of staphylo-

cocci by nurses' clothing. The importance of this will be reported in a separate paper.

The aim of the present investigation was to study the dispersal of *Staph. aureus* from burned patients and the transfer of staphylococci within the ward. The results obtained in this investigation are compared with those of the particle tracer experiments. The influence of high air counts on the nasal carrier rate in the staff has also been studied and an attempt made to evaluate the importance of staphylococcal carriers among the staff as a source of airborne staphylococci.

MATERIALS AND METHODS

Ward design

The design of the ward and its ventilation have been described in detail in an earlier paper (Hambraeus & Sanderson, 1972). The ward is entered via an air-lock with double doors. A corridor runs down the middle of the ward. There are five bedrooms of similar dimensions and a sixth larger room containing an airbed. All these have individual air-locks and they are situated along one side of the corridor. On the other side of the corridor are service rooms and the bathroom. Only burned patients were admitted to the ward. The number of permanent staff working in the ward per week was 25, doctors and night staff included. Protective gowns and masks were used when treating the patients in their rooms. The patients generally did not leave their rooms except for bathing or operations. Weekly reports on the patients occupying the rooms and on bathing and operating schedules were made by the secretary of the ward.

Bacteriology

From the patients specimens were taken from the nose, throat, skin, perineum and wound on admission and thereafter once a week. Weekly specimens from nose and throat were taken from the staff. Deoxyribonuclease-producing staphylococci were classified as *Staph. aureus* (Di Salvo, 1958) and one representative of each morphologically distinguishable type was phage typed (Blair & Williams, 1961). The staphylococcal content of the air was determined by the exposure of settle plates with an inside diameter of 13·5 cm. The medium used was blood agar containing 2 μg./ml. of nalidixic acid to prevent swarming of *Proteus*. The plates were placed three in the corridor and one in each bedroom, 3–5 days a week. From five to nine colonies of *Staph. aureus* found on settle plates in the corridor together with 25 % of colonies (up to a maximum of eight colonies per plate) from settle plates in the bedrooms were phage typed. Strains were assumed to be distributed in the sample in the same ratio as in the colonies that were phage typed. For the determination of the size of the colony-forming units an Andersen sampler (Andersen, 1958) was used.

Methods of calculation

The results of the air count observations in different situations have been presented by plotting the cumulative distribution of the counts on log probability

Table 1. *Number of plates exposed in bedrooms of patients with burns of different extent*

(Median values of staphylococcal dispersal.)

Burned area II + III°	No. of patients	Total no. of plates	Median no.* (c.f.u./m.²/hr.)
⩽ 5 %	12	80	21
6–15 %	17	176	50
16–30 %	15	166	141
> 30 %	11	112	453
Total no.	55	534	—

* The coefficient of variation of each of these values is approximately 20 %.

paper. It has then been assumed that the error of an estimation is largely the Poisson variance. For the Poisson function the values which delimit 50 % probabilities of falling into the lower or upper class are approximately 0·7 above the actual digital value when these are small numbers. It is these limits which have been used when plotting the cumulative distribution.* In all cases it seemed reasonable to draw straight lines through the points plotted in this way, i.e. the distributions were approximately log-normal. The best straight lines were assessed by eye.

RESULTS

The correlation between the extent of the burn and dispersal of Staph. aureus

The individual dispersal of *Staph. aureus* from patients whose burns were colonized with *Staph. aureus* admitted to the ward from February 1969 to September 1971 was determined. Only periods when a patient was treated alone in a bedroom have been analysed. This means that in some cases the period analysed does not consist of consecutive weeks. The patients were divided into four groups according to the extent of the burn. The material is presented in Table 1. Fig. 1 shows the distribution of the air counts of *Staph. aureus* within the four patient groups; 37·5 % of all plates exposed in bedrooms of patients with small burns yielded no staphylococci, the corresponding figure for patients with extensive burns being 4·5 %. The median values varied from 21 c.f.u./m.²/hr. for the least burned patient up to over 450 c.f.u./m.²/hr. for those with the most extensive burns. The maximum dispersal generally occurred during the week of wound colonization or the following week. This was usually the first or second week of the stay in the ward. In most cases the maximum dispersal period did not last for more than one week, except for extensively burned patients, who had longer periods of maximum dispersion. The decrease in dispersal took place whether the patient had been operated upon or not. However, operation always seemed to diminish the dispersal. In three patients a new period of dispersal was noticed late

* This method of plotting the cumulative distribution has been suggested by Dr O. M. Lidwell.

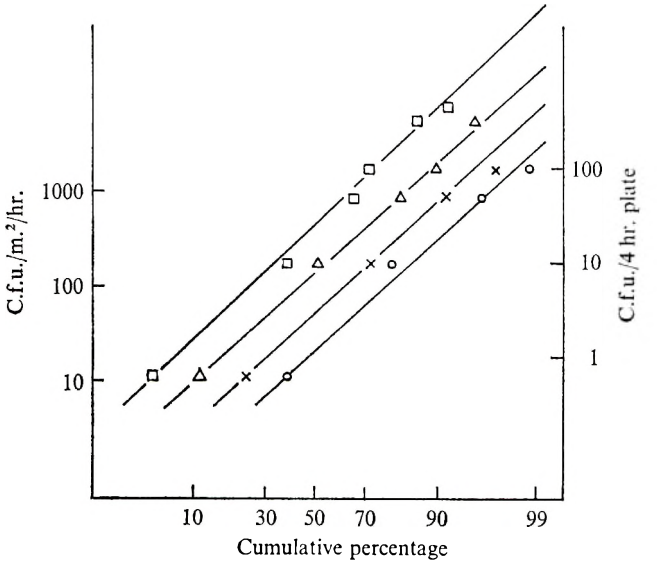

Fig. 1. Dispersal of *Staph. aureus* in relation to burned area. ○, ⩽ 5 % burned area; ×, 6–15 % burned area; △, 16–30 % burned area; □, > 30 % burned area.

Table 2. *Size distribution of* Staphylococcus aureus-*carrying particles according to Andersen sampler*

Week of treatment	No. samples	No. patients	Mean no. *S. aureus*/ m.³	% *Staph. aureus* on different stages of the sampler 1	2	3	4	5	6*	Median particle size (μm.)
1	8	6	339	15·7	22·2	17·6	21·7	18·0	4·8	4·0
2	8	7	78	15·9	16·6	19·0	18·3	25·2	4·9	3·5
3	7	5	81	29·0	16·7	18·3	15·1	17·7	3·2	5·0
> 4	11	6	44	24·9	25·6	12·5	17·6	17·6	1·8	5·6

* Size distribution on stages: 1, ⩾ 9·2 μm.; 2, 9·2–5·5 μm.; 3, 5·5–3·3 μm.; 4, 3·3–2 μm.; 5, 2–1 μm.; 6, < 1 μm.

in the treatment period. This period occurred the same week or the week after the wound was infected with a new staphylococcal strain.

The size distribution of the *Staph. aureus*-carrying particles dispersed was investigated with an Andersen sampler. Thirty-four samples were taken from 19 patients. As far as possible the samples were taken during a period of dispersal, i.e. when the settle plates had shown about 10 c.f.u. in a 4 hr. exposure. The burned area was 15 % or more in all but two cases. Table 2 shows the mean number of *Staph. aureus* per m.³ and the distribution of *Staph. aureus* in the different stages.

The counts found in different weeks of treatment are given. As could be expected, the mean value of *Staph. aureus*/m.³ was highest in samples taken the first week.

The apparent median particle size was much below that suggested by other observations in hospital wards; e.g. Noble, Lidwell & Kingston (1963) found a median particle size of 13–16 μm. The particle size also seems to have been smaller

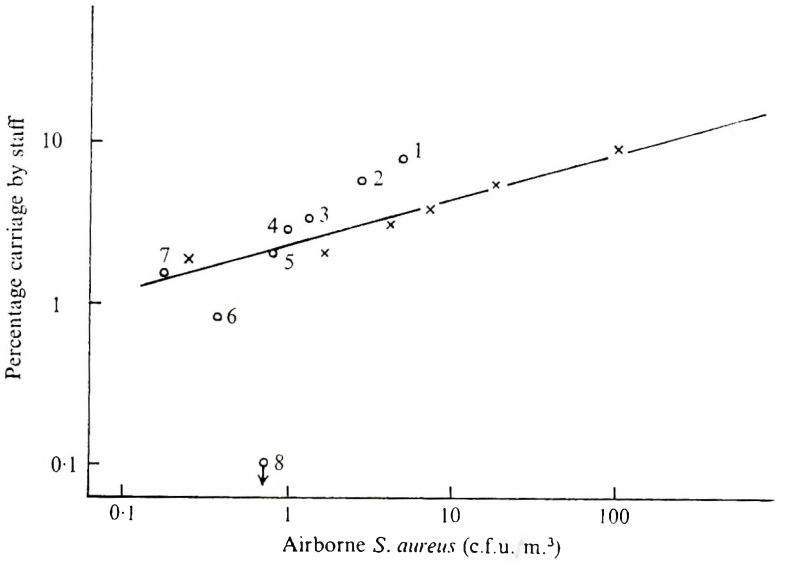

Fig. 2. Relation between nasal carriage by staff and exposure to airborne *Staph. aureus*. Logarithmic scales for both coordinates. Exposure to airborne *Staph. aureus*: mean count/m.³/week assuming a sedimentation rate of 0·3 m./min. (= c.f.u. per 4 hr. plate). The line refers to all strains of *Staph. aureus*. The relationship between exposure and carriage is also given for 8 'epidemic' strains. 1, 84 III (methicillin resistant); 2, NT88; 3, 52/52A/80/81 Complex; 4, 3A II; 5, 53/77/84/85 III; 6, 84/85 III; 7, NT52A/42E/47/53/75/77/88 I + III; 8, 84/85 III (not carried by any member of the staff). 6 and 8 are two strains with the same phage pattern occurring during two separate periods.

early during treatment when the rate of dispersal was greater. The Andersen sampler has been said to be inadequate for collecting larger particles (May, 1964) but a few parallel measurements made in these rooms by Lidwell with the size grading sampler (Lidwell, 1959) also suggested that the particle size was smaller, at least when dispersal was vigorous.

Nasal carriage of Staph. aureus *by the staff*

On an average, six members of the staff per week were nasal carriers of *Staph. aureus*. Over a period of 130 weeks the nasal carrier rate as a function of airborne *Staph. aureus* was studied. This was done in the following way. The mean number of airborne *Staph. aureus* (c.f.u./m.³) of each different phage type found in the bedrooms was calculated for each week from the sedimentation plates, assuming a settle rate of 0·3 m./min. and the number of staff and patients from whom the appropriate type was isolated was recorded. Non-typable staphylococci were excluded. A median of five different phage types was found per week. The sources of airborne staphylococci were: patients only on 296 occasions; staff only on 36 occasions; patients and staff on 181 occasions. No source was found on 128 occasions. The median value of the weekly means was 0·67 c.f.u./m.³ for phage types for which the only known source was patients, 0·06 c.f.u./m.³ for those for which staff carriers were the only source, 1·56 c.f.u./m.³ when both patients and

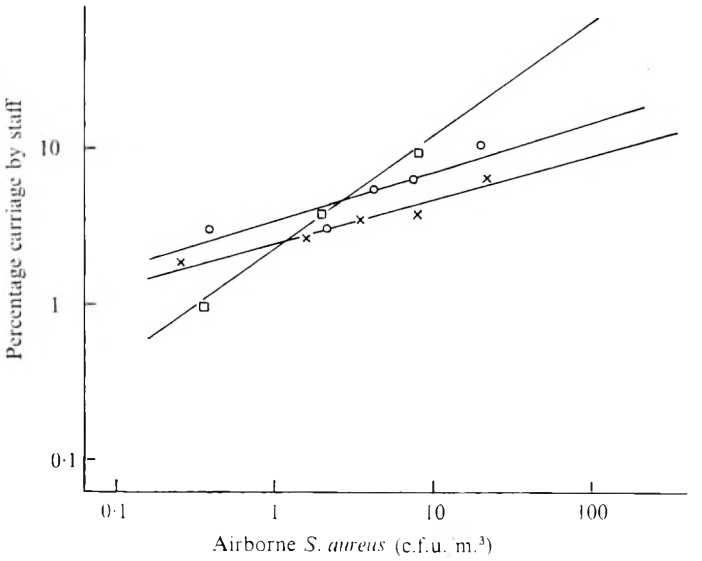

Fig. 3. Relation between nasal carriage of 'epidemic' strains and exposure to airborne *Staph. aureus* for 1, 2 and 3 patient sources. ×, 1 patient source; ○, 2 patient sources; □, 3 patient sources.

staff were possible sources and 0·17 c.f.u./m.³ when there was no recognized source. The proportion of the staff who were carriers of a particular strain is shown in Fig. 2 as a function of the number of that strain per m.³/week for those weeks on which the strain was carried by at least one patient. Nasal carriage was more common when the strain was extensively dispersed. The slope of the line relating the logarithm of the percentage carriage to the logarithm of the extent of airborne dispersal is about 0·30.

An attempt was made to see whether 'epidemic' strains, i.e. those which were present in at least two patients' burns for 6 consecutive weeks or more, differed from the others and whether carriage of the strain by more than one patient showed any effect. The median values for the weekly counts of airborne *Staph. aureus* and the corresponding mean value of the weekly carriage rate for eight such epidemic strains are also shown in the figure. While there was some indication that staff carriage of these strains in relation to the extent of air dispersal was more frequent than with other strains (nos. 1 and 2 in Fig. 2) the effect was not consistent.

The observations recorded with these strains have also been examined in connexion with the number of patient carriers present in any one week, and the results are shown in Fig. 3. Again there is some suggestion that nasal carriage by staff was greater when there were more patient carriers, but this also is not consistent.

The transfer of airborne Staph. aureus *within the ward*

The transfer of staphylococci from a source room to the corridor and to other rooms in the ward was investigated in the following way.

Those occasions were selected when there was a patient dispersing 180 c.f.u./

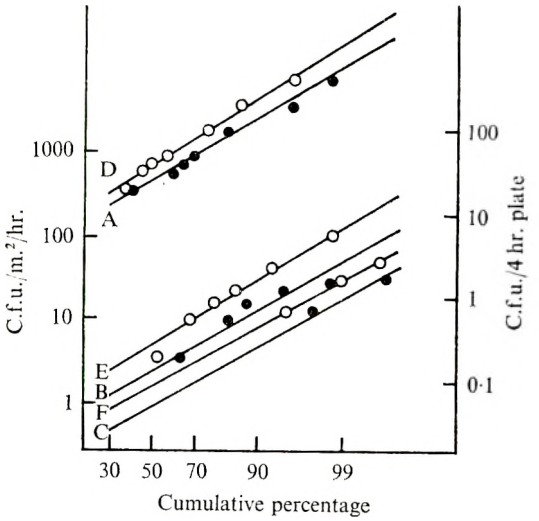

Fig. 4. Comparison of air counts of *Staph. aureus* in dispersers' rooms with counts of the same staphylococci in the corridor and other rooms. Lines A, B and C refer to source room, corridor and receiving rooms respectively, when no staff carriers were present. Lines D, E and F refer to source room, corridor and receiving rooms when staff carriers were present.

m.²/hr. (10 c.f.u./4 hr. plate) or more of a type of *Staph. aureus* which was not isolated from any other patient in the ward. Parallel counts of the number of this type found on settle plates in the source room, i.e. that housing the patient, corridor and other bedrooms were made. If more than one type was dispersed at the same time the different types were considered separately. Days on which the patient was transported from his room into the passage were excluded. Those occasions when the patient was the only source within the ward and those when there were carriers of the phage type among the staff also have been analysed separately. Over a 3-year period 74 occasions without carriers among the staff and 74 occasions with staff carriers were found.

Fig. 4 shows the cumulative distribution of the air counts of the dispersed *Staph. aureus* for the two different circumstances. Lines A, B and C represent source room, corridor and receiving room respectively when no staff carriers were present. Lines D, E and F represent the counts from the same places when staff carriers were present. Median values have been taken from these lines. These were 447 c.f.u./m.²/hr. for source rooms, 2·5 c.f.u./m.²/hr. for the corridor, and 0·9 c.f.u./m.²/hr. for receiving rooms when there were no staff carriers and 708 c.f.u./m.²/hr., 5·0 c.f.u./m.²/hr. and 1·6 c.f.u./m.²/hr. for the same places when staff carriers were present. Although the air counts were slightly higher when staff carriers were present, the ratios of the median values between the three places were similar for both conditions.

Arithmetic mean values were also calculated for the same data. These will be more sensitive to isolated high counts on individual plates. However, if the distribution of the individual dispersion was similar, the ratios between these mean values should be similar to the ratios of the medians. The median and arithmetic

Table 3. *Airborne transfer in the ward*

Situation	Median value (c.f.u./ m.²/hr.)	Arithmetic mean value (c.f.u./ m.²/hr.)	Ratio source room values to other sites: Transfer of *Staph. aureus* Median values	Transfer of *Staph. aureus* Arithmetic mean values	Tracer particle transfer Correct ventilation	Tracer particle transfer Worst ventilation	Tracer particle transfer Average ventilation
A. No staff carriers							
Source room	447	1119	—	—	—	—	—
Corridor	2·5	5·2	179*	215*	$1{\cdot}5 \times 10^3$*	188*	287*
Receiving room	0·9	0·6	497†	$1{\cdot}9 \times 10^3$†	$3{\cdot}4 \times 10^5$†	$4{\cdot}4 \times 10^4$†	$4{\cdot}3 \times 10^4$†
B. With staff carriers							
Source room	708	1498	—	—	—	—	—
Corridor	5·0	11·2	142*	134*	—	—	—
Receiving room	1·6	1·2	443†	$1{\cdot}2 \times 10^3$†	—	—	—

The particle transfer figures for correct ventilation and the worst conditions are those given in a previous paper (Hambraeus & Sanderson, 1972). The average values have been deduced from the proportion of different ventilation conditions observed in the ward (Hambraeus & Sanderson, 1972).

* The ratio concentration in source room/concentration in corridor.

† The ratio concentration in source room/concentration in receiving room.

mean values together with the ratios of the values in the source room to those in the corridor and receiving rooms are given in Table 3. The values for particle tracer transfer (Hambraeus & Sanderson, 1972) are also included for comparison. These values have also been adjusted to allow for the proportion of times when the ventilation system was not functioning correctly (average ventilation). As shown in the table the values for transfer of *Staph. aureus* from source room to corridor are close to those for tracer particles with average ventilation conditions. For transfer from source room to receiving room, however, the ratios source room to receiving room are between 90 and 20 times less than those for tracer particles (average ventilation) depending on whether the median or arithmetic mean values for the counts of *Staph. aureus* are employed.

DISCUSSION

An earlier paper (Hambraeus, 1973) describes the finding that burned patients sometimes disperse large numbers of staphylococci.

This investigation has shown that dispersal is correlated with the extent of the burned area. The median value of dispersal for patients with a burned area of less than 5 % was 21 c.f.u./m.²/hr. and for patients with a burned area of over 30 % it was 453 c.f.u./m.²/hr.

Like air counts found in dermatological wards (Selwyn, 1965), these are very high compared with air counts in ordinary hospital wards where heavy dispersers are seldom found (Noble, 1962; Williams, 1966; Williams & Harding, 1969;

Edmunds, 1970; Lidwell, Polakoff, Davies & Hewitt, 1970; Ayliffe, Collins, Lowbury & Wall, 1971). The correlation of the air counts with the burned area clearly indicates that the burn wound is the most important source of dispersed *Staph. aureus.*

The high air counts of *Staph. aureus*-carrying particles in the ward naturally influenced the rate of nasal carriage of the dispersed *Staph. aureus* among the staff. The slope of the line relating the logarithm of the percentage carriage to the logarithm of the extent of airborne dispersal was about 0·30. If this can be interpreted as the extent to which the staff acquire nasal strains as a result of airborne dispersal by infected patients, it is of some interest to note that the slope is similar to that reported for nasal acquisition of *Staph. aureus* by patients in hospital wards (Lidwell *et al.* 1966, 1970, 1971).

The contribution to the air counts by the staff seemed to be very small; this might of course be partly an artifact as only a small proportion of the *Staph. aureus* found on settle plates in the bedrooms were phage typed. However, it was of the same range as that reported by Edmunds (1970) and settle plates exposed in the corridor and in other areas of the ward never indicated the presence of a disperser among the staff.

The size distribution of the airborne particles carrying *Staph. aureus* was investigated with an Andersen sampler. During periods of dispersion the particles were almost evenly distributed over the five upper stages of the Andersen sampler. The median equivalent particle diameter was 4–5·6 μm., which is less than that found in other wards (Noble *et al.* 1963). Although the Andersen sampler is not completely adequate for sampling larger particles parallel measurements with the Lidwell size grading sampler also indicated that the airborne particles found in the ward were often small.

The transfer of staphylococci in the ward has been compared with the transfer of a particle tracer in an earlier paper (Hambraeus & Sanderson, 1972). These experiments, however, were carried out with a particle size larger than that found for airborne particles in the ward. The contribution of particle sedimentation to the total rate of ventilation in bedroom and corridor and hence its effect on the transfer ratios is given in that paper (loc. cit. p. 306). The effect of smaller particle size and hence lower sedimentation rate can be estimated by reducing the contribution this makes to these ventilation rates; sedimentation in the air-lock is only a minor factor. Elimination of all sedimentation reduces these values by a maximum of 60 %. If we assume a reduction to about 50 % this would seem to be at least as much as is likely for the airborne particle carrying *Staph. aureus* in this unit. The values of the transfer ratios to corridor and receiving room for average ventilation conditions (Table 3) will then be reduced to 144 (one half) and $1{\cdot}1 \times 10^4$ (one quarter) respectively. This is in agreement with experiments with the same particle tracer in a general hospital which showed that transfer of tracer gas between patient rooms situated close to one another was only about three times greater than that of the particles (Foord, 1972).

The transfer of airborne staphylococci within the ward (Table 3) shows reasonable correspondence with the tracer experiments for transfer from the source room

to the corridor, i.e. the results obtained are consonant with the assumption that the airborne particles are carried out into the corridor by air movement. Transfer from the corridor to other bedrooms is, however, as is shown by the overall transfer from source room to receiving room, very much greater than in the case of the particles. Even if allowance is made for the possible effect of a smaller particle size the difference appears to be between 6 and 20 times. This strongly suggests that the apparent transfer from the corridor into these rooms is not due to simple air movement alone. The most likely possibility is that the nurses' clothing becomes contaminated when dealing with an infected patient and that particles are dispersed from this clothing when she enters another patient's room. This would not affect transfer from source room into the corridor as the high levels of air contamination produced in the corridor probably mask this dispersal. Strong evidence that the nurses carry staphylococci from room to room on their clothes in spite of the fact that they put on protective clothing before entering a patient's room, and that these staphylococci are dispersed into the air of the room will be presented in a following paper.

This investigation was supported by grants from the Swedish Delegation for Applied Medical Defence Research.

REFERENCES

ANDERSEN, A. A. (1958). New sampler for the collection, sizing, and enumeration of viable airborne particles. *Journal of Bacteriology* **76**, 471.

AYLIFFE, G. A. J., COLLINS, B. J., LOWBURY, E. J. L. & WALL, M. (1971). Protective isolation in single-bedrooms: studies in a modified hospital ward. *Journal of Hygiene* **69**, 511.

BLAIR, J. E. & WILLIAMS, R. E. O. (1961). Phage-typing of staphylococci. *Bulletin of the World Health Organization* **24**, 771.

EDMUNDS, P. N. (1970). Staphylococcal infection in subdivided general surgical wards. *Journal of Hygiene* **68**, 531.

FOORD, N. (1972). Use of gas and particle tracers in the study of infection transmission. Contribution at the IVth international symposium on aerobiology, Enschede, Netherlands.

HAMBRAEUS, A. (1973). Studies on transmission of *Staphylococcus aureus* in an isolation ward for burned patients. *Journal of Hygiene* **71**, 171.

HAMBRAEUS, A. & SANDERSON, H. F. (1972). Studies with an airborne-particle tracer in an isolation ward for burned patients. *Journal of Hygiene* **70**, 299.

LIDWELL, O. M. (1959). Impaction sampler for size grading airborne bacteria-carrying particles. *Journal of Scientific Instruments* **36**, 3.

LIDWELL, O. M., POLAKOFF, S., JEVONS, M. P., PARKER, M. T., SHOOTER, R. A., FRENCH, I. V. & DUNKERLEY, D. R. (1966). Staphylococcal infection in thoracic surgery: experience in a subdivided ward. *Journal of Hygiene* **64**, 321.

LIDWELL, O. M., POLAKOFF, S., DAVIES, J. & HEWITT, J. H. (1970). Nasal acquisition of *Staphylococcus aureus* in a subdivided and mechanically ventilated ward: endemic prevalence of a single staphylococcal strain. *Journal of Hygiene* **68**, 417.

LIDWELL, O. M., DAVIES, J., PAYNE, R. W., NEWMAN, P. & WILLIAMS, R. E. O. (1971). Nasal acquisition of *Staphylococcus aureus* in partly divided wards. *Journal of Hygiene* **69**, 113.

MAY, K. R. (1964). Calibration of a modified Andersen bacterial aerosol sampler. *Applied Microbiology* **12**, 37.

NOBLE, W. C. (1962). The dispersal of staphylococci in hospital wards. *Journal of Clinical Pathology* **15**, 552.

NOBLE, W. C., LIDWELL, O. M. & KINGSTON, D. (1963). The size distribution of airborne particles carrying micro-organisms. *Journal of Hygiene* **61**, 385.

Di Salvo, J. W. (1958). Deoxyribonuclease and coagulase activity of micrococci. *Medical Technicians Bulletin* **9**, 191.

Selwyn, S. (1965). The mechanism and prevention of cross-infection in dermatological wards. *Journal of Hygiene* **63**, 59.

Williams, R. E. O. (1966). Epidemiology of airborne staphylococcal infection. *Bacteriological Reviews* **30**, 660.

Williams, R. E. O. & Harding, L. (1969). Studies of the effectiveness of an isolation ward. *Journal of Hygiene* **67**, 649.

J. Hyg., Camb. (1973), **71**, 799
With 2 plates
Printed in Great Britain

Transfer of *Staphylococcus aureus* via nurses' uniforms

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(*Received* 9 *April* 1973)

SUMMARY

The contamination of gowns and uniforms worn in a burns unit and the transfer of patient's staphylococci by means of nurses' uniforms was investigated. The median values of staphylococci found on gowns and jackets worn during a routine nursing procedure were $3{\cdot}0 \times 10^4$ and $1{\cdot}4 \times 10^3$ respectively. From the results of model nursing experiments it appeared as if the fraction of staphylococci transferred from a patient's room to the air in a receiving room was 4 to 10 times less when protective gowns were worn than when no gowns were worn. The corresponding transfer directly to the model 'patient' was 100 times less. The protection afforded by a gown seemed mainly to be due to protection against contamination of the uniform worn underneath when nursing a burned patient. The discrepancy between the transfer of an airborne particle tracer and *Staph. aureus*-carrying particles earlier found in the ward could be explained by the dispersal of *Staph. aureus* from nurses' clothing.

INTRODUCTION

There is evidence that bacteria-carrying particles rather easily pass from the surgeon's skin into the air when a conventional surgical outfit is worn (Duguid & Wallace, 1948; Hare & Thomas, 1956; Bethune, Blowers, Parker & Pask, 1965; Charnley & Eftekhar, 1968; Sykes, 1970), and several workers have tried to diminish this source of infection in operating rooms by introducing new materials as well as by altering the design of the outfit (Blowers & McCluskey, 1965; Bernard, Cole & Gravens, 1967). Although it has been shown that nurses' clothing becomes contaminated with staphylococci common in the ward during work (Speers *et al.* 1969; Lidwell & Towers, 1972), the importance of this has been very little studied. It may well be that this is an important means of secondary transmission in ordinary ward work as well as in barrier nursing when common protective gowns are worn.

In a previous investigation in a burns ward (Hambraeus, 1973) it was shown that the transfer of staphylococci within the ward was much greater than the transfer of an airborne tracer particle. This indicates that transfer of *Staph. aureus* is not only due to air movement.

The aim of the present investigation was to study the importance of transfer of *Staph. aureus* by means of nurses' clothing in this ward.

The contamination of protective gowns and the uniform worn underneath during work in the ward has been studied. The effectiveness of four different kinds of commercially available protective gowns has been investigated experimentally. An attempt to measure the patient-to-patient transmission of staphylococci by means of nurses' clothing has been made.

MATERIALS AND METHODS

Ward design

The ward has been described in detail in an earlier paper (Hambraeus & Sanderson, 1972). It has five bedrooms and a 6th room containing an airbed, all with individual air-locks. The ventilation rate in the bedrooms is about four air changes/hr.

Uniforms and nursing routines

The uniform used in the ward consisted of a short-sleeved cotton jacket and cotton trousers (Plate 1). When nursing a newly burned patient a cotton surgical gown (Plate 2) was used. This was also used with a plastic apron underneath when bathing a patient. In all other nursing procedures a semisynthetic protective gown was used. The nurses put on a gown in the air-lock before entering the room. The gowns were kept in the air-lock and changed once a day. Gloves were used in all nursing procedures. The morning nursing procedure generally lasted for from 30 to 70 min. It included washing the patient, bed-making and tidying the room. The floor was cleaned by staff from the central cleaning department common to the whole hospital.

Sampling techniques

Among the most common methods used when sampling bacteria from fabrics are the sweep plate-, contact plate-, and vacuum sampling methods (Blowers & Wallace, 1955; Williams & Shooter, 1963; Hall & Hartnett, 1964; Robinton & Mood, 1968; Nicholes, 1970). These three methods were compared on 13 protective gowns contaminated in ordinary ward work. Two sweep plates, six contact plates (Rodac plates) and two plates from vacuum samplings (Casella slit sampler, air flow 150 l./min.) each run for 2 min. were used on the front part of each gown. Using the vacuum sampling method about 10 times as many c.f.u. of *Staph. aureus* were isolated as by the two other methods. As shown in Table 1 about twice as many strains with different phage patterns were also found by this method.

All these methods only sample a portion of the possible micro-organisms on the gown and a fourth method, the wash method, was therefore introduced. Two litres of 1% peptone water were used for each washing. A litre of the wash fluid was filtered through millipore filters (0·45 μm., 47 mm.), using 5–10 filters per litre, and bacteria were eluted from the filters by shaking them with glass beads in 10 ml. nutrient broth. Colony counts were made on 0·25 up to 2·5 ml. of the 10 ml. lots. The volumes chosen depended on the assumed contamination of the gown. There were very small variations in the number of staphylococci found in each filtered portion from the same wash fluid. At first washing was done by hand,

Table 1. *Number of strains with different phage patterns isolated by three different sampling methods*

	Strains found	% of max.*
Sweep plates	16	34
Rodac plates	18	38
Vacuum sample	34	72

* i.e. % of the total number of strains recovered by all three methods used simultaneously.

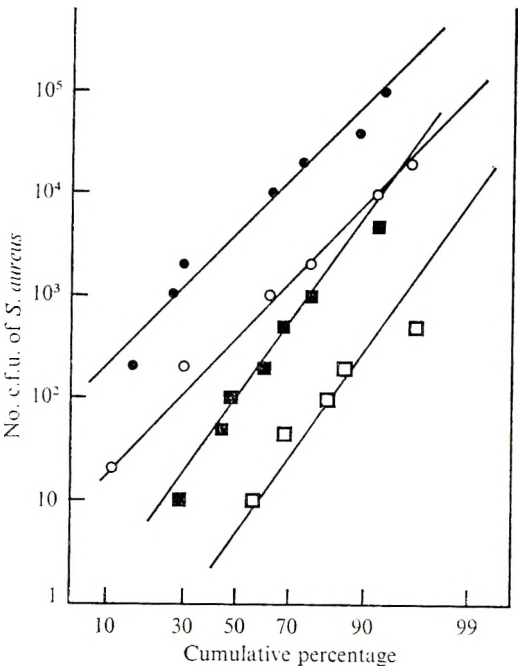


Fig. 1. Distribution of total number of *Staph. aureus* found on jacket and gown by vacuum sampling method and wash method. Number *Staph. aureus*: number c.f.u. per single wash or per two vacuum sample plates.

■ Protective gown
□ Jacket
} Vacuum sampling method

● Protective gown
○ Jacket
} Wash method

always by the same person; later a small washing machine in which the drum was replaced by a special autoclavable drum was used. There were no important differences in the results from the two washing methods.

In order to compare the vacuum method and the wash method, 28 pairs of gowns and jackets worn during the morning nursing of patients were investigated by the two methods. Fig. 1 shows the distribution of the total number of *Staph. aureus* found by the two methods. The median number of *Staph. aureus* colonies found in samples from protective gowns was $4{\cdot}0 \times 10^3$ from a single wash and from the jackets $4{\cdot}0 \times 10^2$ per single wash. With the vacuum sampling method the

Table 2. *Number of strains with different phage patterns isolated from protective gowns and jackets by vacuum sampling method and wash method*

	Protective gown		Jacket	
	Strains found	% of max.*	Strains found	% of max.*
Vacuum sample	76	65	67	54
Wash method	92	79	103	82

* i.e. % of the total number of strains recovered by the two methods used simultaneously.

median values were 1×10^2 and five per two slit sampler plates for gowns and jackets respectively. The larger values found in the wash fluids probably result in part from dispersal of clumps of organisms. However, as is shown in Table 2, more strains with different phage patterns were also collected by the wash method. Being the most effective method and the one showing a lower variability, the wash method was adopted for the investigation. Six consecutive washings of seven gowns and four jackets showed that only a portion of the micro-organisms on a piece of cloth was recovered in 2000 ml. wash fluid by a single wash. Although the results from repeated washings were somewhat variable, about 56 % were recovered from a jacket and about 38 % from a gown. The washing of the gowns was probably less efficient because they were larger. To estimate the total number of *Staph. aureus*, the number recovered in 1000 ml. wash fluid (the portion filtered) from the jacket has been multiplied by $2 \times 1{\cdot}8$, and the number recovered from the same volume from a gown or bottom sheet has been multiplied by $2 \times 2{\cdot}7$. In what follows it is these estimated total numbers of *Staph. aureus* that are given.

Methods for measuring the transfer of Staph. aureus*-carrying particles through protective gowns*

(1) Transfer through gowns worn in normal work was examined using pairs of protective gowns and jackets worn during a routine morning nursing of a patient. Before entering the room the nurse put on sterile jacket and trousers and a sterile gown. After the nursing procedure the jacket and the gown were taken off the nurse and put in different plastic bags for transport to the laboratory where they were immediately washed.

(2) Pairs of gowns and jackets worn during a standardized exercise were examined in the following way: a standardized exercise, 75 golf strokes, was performed by a laboratory assistant. The protective gown being tested was worn over a sterile uniform (jacket and trousers). A contaminated gown was put on (inside out) over the combination. The contaminated gown had been used in the ward so that the contamination was representative of that produced by use. After the experiment the contamination of the gown being tested and the jacket was determined by the wash method. As long as the test person was not a *Staph. aureus* carrier no phage typing was done. When she became a carrier of staphylococci, all staphylococci from the jacket and all up to a maximum of 8 per colony count plate from the barrier gown were phage typed.

Table 3. *Materials used for protective gowns*

Name	Fibre	Weight/m.^{2}	Threads/cm.	% Transmission 0·5–1 μm.	% Transmission 1–10 μm.
Cotton gown	100 % cotton	165	19 × 25	58	50
Poplin gown	65 % polyester 35 % cotton	160	42 × 22	57	47
Bar-Bac gown	100 % cotton	187	38 × 31	75	58
Disposable gown	50 % rayon 50 % cellulose	60	non-woven	66	54

Methods for estimating the transfer of Staph. aureus *from one patient to the other by means of nurses' clothing*

In these experiments a nurse dressed in sterile jacket and trousers and a sterile protective gown performed the routine nursing of a patient. She then undressed and the jacket and trousers were put on by a laboratory assistant who carried out a model nursing procedure with a volunteer from the laboratory acting as patient. The model nursing procedure resembled the real nursing routine as closely as possible. Thus the laboratory 'nurse' also wore a sterile gown, sterile gloves, cap, mask, and shoe covers. The 'patient' wore a sterile protective gown, sterile gloves, cap, mask, and shoe covers. The experimental nursing procedure lasted for about 25 min.

The air contamination during the model nursing procedure was investigated using a Casella slit sampler with an air flow of 700 l./min. This was run for five consecutive periods of 5 min. each, i.e. a total of 25 min. during each nursing period. Samples were also taken before each experiment. The contamination of the jacket, the protective gowns used by the volunteer 'nurse' and 'patient' and the sterile bottom sheet which was used during the performance was investigated by the wash method.

Characterization of the material of the protective gowns used in the investigation

Four different kinds of protective gowns were used; a cotton surgical protective gown of the kind routinely used in the hospital, a polyester-cotton (poplin) gown, also in routine use, a closely woven cotton (Bar-Bac)* surgical protective gown, and a non-woven disposable surgical protective gown. These will be referred to as cotton gown, poplin gown, Bar-Bac gown and disposable gown respectively. Fibre weight/m.^{2}, threads/cm. and % transmission of particles are given in Table 3. Percentage transmission refers to an investigation in which room air was sucked through the fabric and the difference in particle concentration in filtered and non filtered air measured with a Royco particle counter.†

* Angelica Uniform Company, New York.

† Performed by B. Martensson, Stora Kopparbergs specialprodukter.

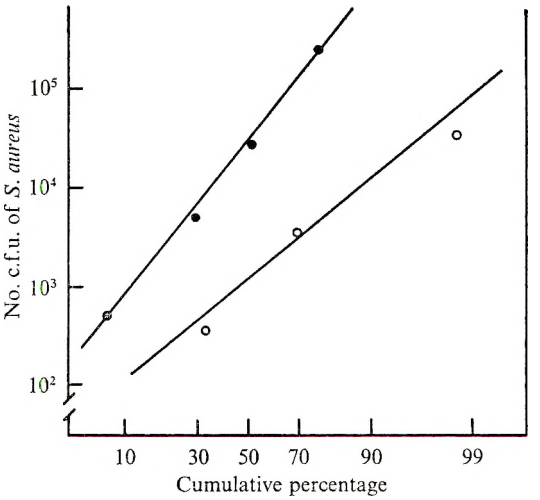


Fig. 2. Distribution of total number of *Staph. aureus* on gowns and jackets worn in routine nursing. ●, Protective gown; ○, jacket.

Bacteriological methods

Sedimentation plates were exposed in the patients' rooms 3–5 days/week. From the patient, samples were taken from the upper respiratory tract, skin, perineum and wound once weekly and, in most cases, also on the day of the experiment. Samples from the upper respiratory tract of all persons taking part in the experiment were taken on the day of the experiment. Samples were taken from the hands of the volunteer 'nurse' and 'patient' after each experiment. Cultures were made on blood and phenol-mannitol agar. Blood agar was used for the air and textile sampling plates. The plates were incubated for 48 hr. at 37° C. Presumptive *Staph. aureus* colonies were tested for deoxyribonuclease production (Di Salvo, 1958). From human specimens, one representative of each morphologically distinguishable type was phage typed (Blair & Williams, 1961). All *Staph. aureus* colonies up to a maximum of eight found on each plate from the textile samples and all colonies from the air samples taken during experiments were phage typed, except in a few experiments with an extremely high air contamination. Strains were assumed to be distributed in the sample in the same ratio as in the colonies that had been phage typed.

RESULTS

Transfer of Staph. aureus-*carrying particles through protective gowns*

Contamination of the protective gown and of the jacket worn underneath, during routine nursing procedures

Fifty-seven pairs of protective gowns and jackets that had been worn during routine nursing of a patient were examined. Both gowns and jackets had been sterilized before use and were worn over the nurses' own underclothing. Cotton, poplin and disposable gowns were used, but as there were no measurable differences between them the results have been combined. Fig. 2 shows the distri-

Table 4. *Number of gowns and jackets from which patients' and staff strains of staphylococci were isolated*

Source of *Staph. aureus*	Protective gowns		Jackets	
	No.	% of total no. *Staph. aureus*	No.	% of total no. *Staph. aureus*
Patient	19	89	15	54
Staff	4	0·07	8	2
Other*	19	11	20	44
Total no.	21	—	21	—

* These were often strains present in the ward carried by other patients or staff.

bution of the estimated total number of *Staph. aureus* recovered from the gowns and from the jackets. The median values were $3{\cdot}0 \times 10^4$ and $1{\cdot}4 \times 10^3$ for protective gowns and jackets respectively.

Phage-typing was carried out in 25 of the pairs investigated. Table 4 shows the result of this. In four cases patient and staff carried *Staph. aureus* with the same phage type. These cases have been excluded. From 19 of the protective gowns and 15 of the jackets strains with the same phage type as those isolated from the patient were found. The contribution of these strains to the total number of staphylococci recovered varied considerably. 89 % of the total number of *Staph. aureus* derived from the protective gowns were patient strains. From the jackets the corresponding figure was 54%. On four of the protective gowns and on eight of the jackets strains with the same phage types as those isolated from the upper respiratory tract of the staff were found. The proportion of these to the total number of colonies isolated was only 0·07 % on the gowns and 2 % on the jackets. Strains with phage patterns other than those of patient or staff strains were often recognizable as strains carried by other persons in the ward. The ratio of the number of *Staph. aureus* found on the protective gown to that found on the jacket was scattered over a wide range (from less than 1 to over 1000). For patient strains the median value of the ratio was 25.

Barrier effect achieved with different kinds of protective clothing worn during a standardized exercise

Four different kinds of protective clothing were used during standardized exercise as described. These were cotton gown, poplin gown, disposable gown and Bar-Bac gown (Table 3). Ten gowns of each fabric, all new and never laundered before, were tested two to three times each. In an additional series a plastic apron was worn underneath the cotton gown and in another a plastic bag was worn on top of the jacket, underneath a cotton gown. These combinations of clothing were tested 20 times each. In Table 5 the transmission of staphylococci is shown expressed as the proportion of staphylococci on the jacket to that on the barrier gown. There were rather small differences but the list order for the four materials was the same as that found for transmission of 1–10 μm. particles. Neither the plastic apron nor the bag showed any large effect.

Table 5. *Comparison of transmission through gowns*

Name	Median (%)	Quartiles	% transmission of particles (1–10 μm.)
Cotton gown	1·6	0·7–3·5	50
Poplin gown	0·7	0·5–1·8	47
Bar-bac gown	2·2	1·0–4·4	58
Disposable gown	2·4	1·7–5·4	54
Cotton gown + plastic apron	1·8	0·3–3·5	—
Cotton gown + plastic bag	0·6	0·2–0·9	—
Gowns used in routine nursing	4·0	0–6·3	—

Estimation of the transfer of Staph. aureus *from one patient to the other by means of nurses' clothing*

An attempt to quantitate the transfer of *Staph. aureus* from one patient to the other by means of nurses' clothing was made using model nursing experiments performed as described. In 15 nursing experiments cotton gowns were used by both the ward nurse and the person nursing the experimental 'patient', in 15 experiments Bar-Bac gowns were used by the two and in 15 experiments no protective gowns were used either by the ward nurse or by the volunteer 'nurse'.

Contamination of jackets during patient nursing

The jackets were heavily contaminated during the nursing procedure. The median value of the total number of *Staph. aureus* found was $9{\cdot}0 \times 10^3$ when cotton gowns and $1{\cdot}8 \times 10^3$ when Bar-Bac gowns were worn. These median values were slightly higher than those given earlier for the contamination of jackets worn during nursing procedures. This was probably due to the fact that as far as possible heavily dispersing patients were chosen for the model nursing experiments. When no protective gown was worn the median value was $1{\cdot}1 \times 10^5$. The majority (95, 70 and 97 % respectively) of the staphylococci were of the same phage type as those isolated from the patient.

In 36 experiments the ward nurse was a carrier of *Staph. aureus* and in 26 of these it was of another phage type than those isolated from the other participants in the experiment, in eight experiments it was the same phage type as the burned patient's, and in two the ward nurse and the volunteer 'patient' had *Staph. aureus* with the same phage type. In those experiments in which the ward nurse carried an easily distinguishable *Staph. aureus* this type was isolated from the jacket in four cases; the amount varied from 12 to 1·4 % of the total number of *Staph. aureus* found. When the ward nurse and the patient were infected with the same *Staph. aureus* type, those found on the jacket were assumed to be the patient's, as consecutive nursings of the burned patient performed by carriers and non-carriers gave the same results. The volunteer 'nurse' and 'patient' were intermittent carriers of *Staph. aureus*; these were usually easily distinguishable from the other *Staph. aureus* types isolated and they were very seldom dispersed.

In Fig. 3 the relation between the degree of air contamination in the real

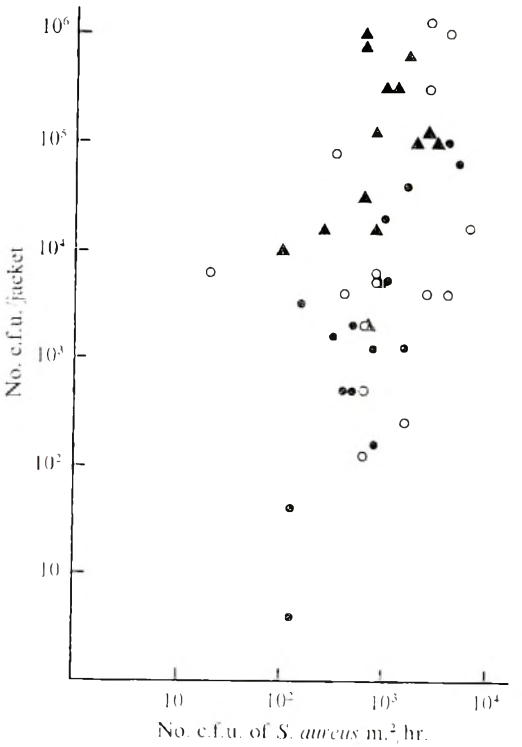


Fig. 3. Comparison between air counts in the patient's room (c.f.u./m.^{2}/hr.) and contamination on the jacket. ○, Jacket worn under cotton gown; ●, jacket worn under Bar-Bac gown; ▲, jacket worn without gown. Fifteen experiments each.

patient's room and the number of this patient's staphylococci found on the jacket is shown, the air count serving as an index of the level of contamination to which the nurse was exposed. In a few cases settle plates were not exposed in the patient's room the day of the model nursing, the mean number of the observations on the day before and after was then used. To estimate the protection against contamination given by the gowns log median values for air contamination in the patient's rooms and on the jackets were determined graphically by plotting the cumulative distribution; the median values, assuming a sedimentation rate of 0·3 m./min., were 56, 32 and 45 c.f.u./m.^{3} in the patients' rooms when cotton gown, Bar-Bac gown and no gown was worn. The corresponding median values for the jackets were $8{\cdot}5 \times 10^3$, $1{\cdot}6 \times 10^3$ and $5{\cdot}6 \times 10^4$ c.f.u. *Staph. aureus*. The log median values together with the ratio of counts on the jackets to those in the air, expressed as the log difference, are given in Table 6. The log difference when cotton gown was worn is 0·9 less than that when no gown was worn, the log difference when Bar-Bac gown was worn is 1·4 less than that for no gown worn; this would indicate that wearing a gown gives an 8 to 25 fold protection against contamination of the jacket.

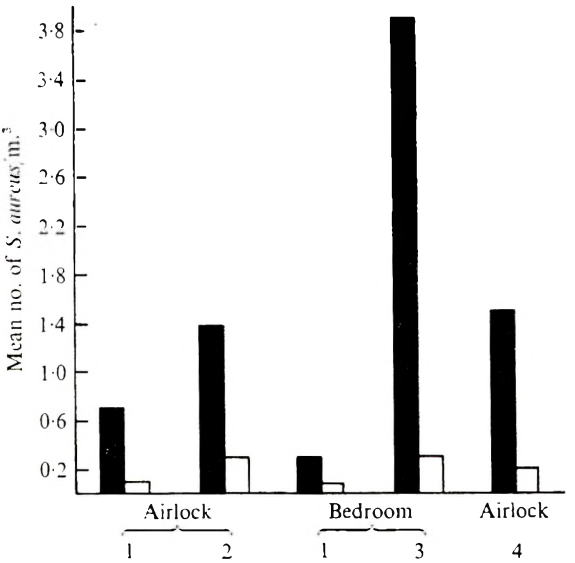


Fig. 4. Mean number c.f.u. *Staph. aureus*/m.³ in air-lock and bedroom during nursing experiments. Black column: *Staph. aureus* with the same phage type as those isolated from the jacket. White column: other staphylococci. 1, no activity; 2, dressing in the air-lock; 3, model nursing; 4, undressing in the air-lock.

Transfer of Staphylococci to the receiving room

Dispersal to the air. The air contamination in the bedroom during model nursing, and in the air-lock to this room during dressing and undressing, for the series of experiments in which both real and model nursing was performed in cotton gowns is shown in Fig. 4. The values given for dressing and undressing are mean values of 12 and 11 investigations respectively, the bedroom values are mean values of 15 investigations. The numbers of staphylococci with phage types other than those isolated from the jackets are fairly constant, but the numbers of staphylococci derived from the jackets are high during activity; the mean number of these found in the bedroom is 3·9/m.³ and in the air-lock it is 1·4/m.³. As shown in the figure, staphylococci with the same phage type as those isolated from the jacket were sometimes found in the air, especially in the air-lock before the experiments had begun. These staphylococci were often of a phage type isolated from more than one person in the ward.

In order to analyse more closely the transfer of staphylococci from a patient to a receiving room, those staphylococci were chosen that had the same phage type as those isolated from the burned patient but which were not present or present only in small numbers in air samples in the receiving room before the experiments. These staphylococci will be called 'marker' staphylococci. In Fig. 5 the air counts in the burned patients'rooms are compared with those found in the receiving room during model nursing. (The same method for plotting the cumulative distribution has been used as that given in an earlier paper, Hambraeus, 1973.)

The median values for the air counts in the receiving room were 0·22, 0·09 and 0·89 c.f.u./m.³, when cotton gowns, Bar-Bac gowns and no gowns were worn. The

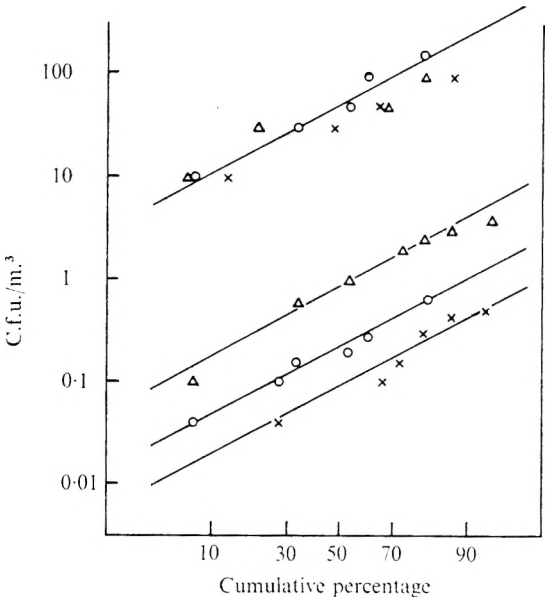


Fig. 5. Distribution of the air counts in patients' rooms and in the receiving rooms during model nursing. The number of *Staph. aureus* /m.³ given assumes a sedimentation rate of 0·3 m./min. The upper line refers to air counts in patients' rooms. Because of the closeness of the values for the patients' rooms, only one line is drawn. The lower lines refer to air counts in the receiving rooms during model nursing. ○, Experiments in which cotton gowns were worn; ×, experiments in which Bar-Bac gowns were worn; △, experiments in which no gowns were worn.

log median values as well as the ratio of the air counts in the receiving room to those in the patient's room, expressed as the log difference, are given in Table 6. The percentage *Staph. aureus* carrying-particles transferred varies from about 2 when no gown was worn to between 0·4 and 0·3 when a cotton gown or a Bar-Bac gown was worn, i.e. there was approximately a five- to seven-fold improvement on wearing a gown. In Table 6 the air counts in the receiving room are also expressed as a function of the air counts on the jackets, i.e. the log difference of the counts in the receiving room to those on the jackets are given. From these figures it appears that there was no protective effect of wearing the gowns, in fact the log difference seems to be smallest when no protective gown was worn. This indicates that the gowns did not reduce the dispersal of organisms from a contaminated uniform.

In three cotton gown experiments the dispersion was extremely high 8·9, 9·8, and 36·5 c.f.u./m.³. In one of these experiments the volunteer 'patient' was heavier than in the other experiments; in two the burned patient was a little girl dispersing heavily. Staff were carriers of an easily distinguishable phage type in 26 experiments; these were isolated from air samples in six cases. The median value of these six was 0·06 c.f.u./m.³ and the maximum value was 0·1 c.f.u./m.³. This was found in one of the experiments without protective gowns.

Transfer of Staph. aureus *to the model 'patient'*. Fig. 6 shows the comparison between the number of 'marker' *Staph. aureus* found on the jackets and those

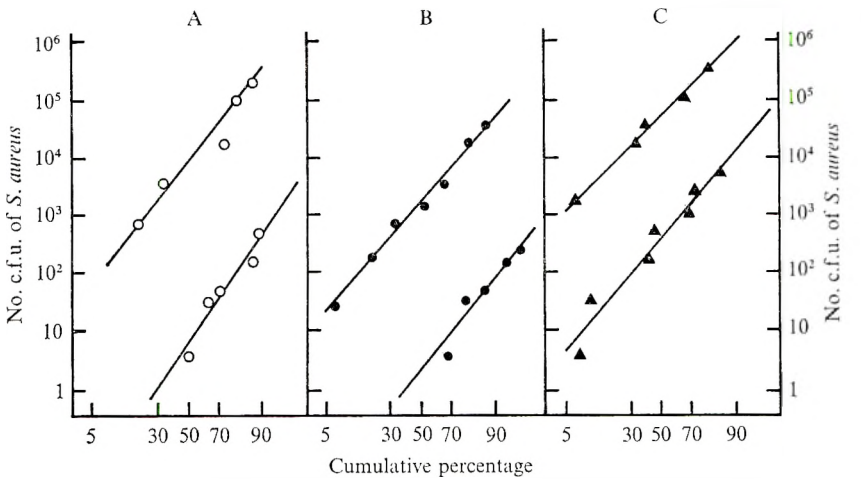


Fig. 6. Comparison between the number of 'marker' staphylococci found on the jackets and those found on the model 'patients'' gowns and bottom sheets. The upper line shows the distribution of the number of 'marker' *Staph. aureus* found on the jackets: the lower line shows the distribution of the number of 'marker' *Staph. aureus* found on the model 'patients'' gowns and bottom sheets. A, Experiments in which cotton gowns were used (○); B, experiments in which Bar-Bac gowns were used (●); C, experiments in which no gowns were used (▲).

found on the model 'patients'' gowns and bottom sheets after the model nursing procedure when cotton gowns, Bar-Bac gowns and no gowns were worn. Median values for 'patient's' gown and bottom sheet were 6, 2 and 4×10^2 c.f.u. and maximum values were 3×10^3, 7×10^2 and 2×10^4 respectively. Two items in the series with gowns had to be excluded, one because the model 'patient' had marker staphylococci on her hands after the experiment and the other because the model 'patient' and the burned patient carried staphylococci with the same phage type. To estimate the protection against contamination of the patient afforded by the gowns, the ratios between the counts on the model 'patients'' gown and bottom sheet and those in the burned patient's room were calculated for the 3 different situations. The log difference between the median values are given in Table 6. The log difference when cotton gowns were worn is 1·88 less than that when no gowns were worn; the log difference when Bar-Bac gowns were worn is 2·12 less than that when no gowns were worn, this seems to indicate that wearing either gown reduced the transfer of *Staph. aureus* to the patient by about 75 to 130 times. The log differences between the number of staphylococci found on the model 'patient's' gown and bottom sheet and those on the jacket has also been calculated. There is some evidence that wearing either gown reduced transfer from the jacket to the patient but the effect was not very great, a reduction of between about 10 to 5 times (log difference when cotton gown was worn being 0·96 less than that when no gown was worn, log difference when Bar-Bac gown was worn being 0·72 less than when no gown was worn).

Table 6. *Transfer of marker staphylococci in model nursing experiments*

	Gowns used			Log difference between not wearing and wearing	
	Cotton gown	Bar-Bac gown	No gown worn	(*a*) Cotton gown	(*b*) Bar-Bac gown
Air count patient's room (log med c.f.u./m.^{3}*)	1·75	1·50	1·65	—	—
Counts on jacket (log med c.f.u.)	3·93	3·20	4·75	0·82	1·55
Ratio counts on jacket–air count patient's room (log difference)	2·18	1·70	3·10	0·92	1·40
Air count receiving room (log med c.f.u./m.^{3} + 6)	5·35	4·94	5·95	0·60	1·01
Ratio air count receiving room–air count patient's room* (log difference + 6)	3·60	3·44	4·30	0·70	0·86
Ratio air count receiving room–counts on jacket (log difference + 6)	1·42	1·74	1·20	− 0·22	− 0·54
Counts on model patient† (log med c.f.u. + 4)	4·77	4·28	6·55	1·78	2·27
Ratio counts on model patient†–air count in patient's room* (log difference + 4)	3·02	2·78	4·90	1·88	2·12
Ratio counts on model patient†–counts on jacket (log difference + 4)	0·84	1·08	1·80	0·96	0·72

* Assuming a sedimentation rate of 0·3 m./min.
† Protective gown and bottom sheet.

DISCUSSION

The aim of this investigation was to obtain an estimate of the degree of contamination of nurses' clothing with *Staph. aureus* during work in the ward and to calculate the risk of transfer of *Staph. aureus* from room to room by means of nurses' clothing. For the isolation of *Staph. aureus* from the textiles four different sampling techniques were tried; sweep-plates, Rodac plates, vacuum sampling and the wash method. Of these, the wash method was the most effective. One of the advantages of this method was that the whole item could be investigated. Some of its effectiveness might be due to the fact that *Staph. aureus*-carrying particles were split during the washing procedure. However, the method was also more effective in recovering different strains of *Staph. aureus*.

It was shown that the clothes, both the protective gown and the jacket worn underneath, were heavily contaminated during the nursing of a burned patient. The proportion of staphylococci that penetrated the protective gown during a nursing period varied considerably. This might be due to differences in the work performed by the nurse according to whether an extensively burned patient, a baby or a nearly well patient was treated. Because of these variations it would not have been possible to compare the protection given by different kinds of protective gowns by using them in routine work.

An attempt to investigate the barrier effect of six different kinds of protective clothing by using them in a standardized pattern of movement was therefore made. Only very slight differences in protection could be shown, which was in agreement with the results achieved with a particle penetration test. The Bar-Bac fabric did not seem to be better than the other fabrics used. This is not consonant with the findings of other authors (Bernard, Cole & Gravens, 1967; Michaelsen, Halbert, Sorensen & Vesley, 1968). There is probably not much difference between the transmission through the fabrics if the particles are small and this may explain the results. The range of particles in room air is probably more near 1 μm. than 10 μm. and it has been shown in an earlier paper (Hambraeus, 1973) that burned patients often disperse a portion of small bacteria-carrying particles. The best results were achieved when a plastic bag was worn on top of the jacket and under the barrier gown.

In the model experiments on transfer of *Staph. aureus* from a burned patient to a receiving room, cotton gowns, 'Bar-Bac' gowns and no protective gowns were used, each in 15 experiments. The results from these experiments are summarized in Table 6. From the log median values it appears as if the dispersal of staphylococci into the receiving room was 4 to 10 times less and the transfer of staphylococci to the model 'patient' about 100 times less when protective gowns were used than when no gown was used. This effect, however, seems to be mainly due to protection against contamination of the jacket when nursing the burned patient. When the number of air borne staphylococci in the burned patient's room served as an index of the level of contamination to which the nurse was exposed then this protection seemed to be 8- to 25-fold. When worn over a contaminated uniform, neither of the two gowns appeared to have any effect in reducing the dispersal of the contaminating organisms into the air of the receiving room. This may have been because of a greater release of bacteria-carrying particles from the jacket due to friction between jacket and gown; similar results have been found by other authors (Bethune, Blowers, Parker & Pask, 1965). There did seem to be a rather small reduction in transfer to the model patient's clothes and bed linen when the gowns were worn, compared with transfer during the same procedures performed without a gown.

As the cotton gown was the one used when nursing newly burned patients in the ward, the results of this series of experiments are the most interesting from the epidemiological point of view. The median airborne dispersal of 0·22 c.f.u./m.^{3} during an experimental nursing period of 25 min. would correspond to a settling of 1·7 c.f.u./m.^{2} during this time. The maximum value achieved during an experiment was 36·5 c.f.u./m.^{3}. This would correspond to a settling of 273·8 c.f.u./m.^{2} during a nursing period. The real nursing of a patient generally takes slightly longer than the model nursing, and the staff probably moves around a little more; this together with the fact that the protective gowns used in a bedroom were only changed once a day unless they were visibly dirty, and the jacket and trousers were worn throughout the day, probably means that the real dispersal from clothing in the ward would be somewhat higher than the median value found experimentally.

In an earlier investigation (Hambraeus, 1973) it was shown that the fraction of

Staph. aureus-carrying particles transferred from a source room to a receiving room was $0{\cdot}5 \times 10^{-3}$, whereas the fraction of an airborne tracer particle transferred was $0{\cdot}17 \times 10^{-4}$. The median value of *Staph. aureus*-carrying particles found in a receiving room was 0·9 c.f.u./m.^{2}/hr. This was calculated from the settling of *Staph. aureus* on plates exposed for 4 h. Assuming that there is one nursing procedure during a plate exposure, 1·7 c.f.u./m.^{2} would be equivalent to 0·43 c.f.u./m.^{2}/hr. As there would also be other nursing activities during the 4 hr. period, this figure is clearly of the same order as that of the median value of *Staph. aureus*-carrying particles found in the receiving room. Thus dispersion from clothing could account for the whole discrepancy found between the transfer of *Staph. aureus* and that of airborne tracer particles in the ward.

Besides the presence of airborne staphylococci dispersed from clothing, it was also possible to isolate staphylococci derived from the burned patient on the protective gown and bottom sheet used by the volunteer 'patient'. In only three experiments were no staphylococci found on the protective gown or bottom sheet; the maximum value found was 3×10^3 c.f.u.

Little is known about the infective dose of staphylococci. Shinefield *et al.* (1963) could set up a carrier state in the nose of 50 % of newborn infants by the inoculation of between 200 and 400 cocci. In experimental colonization of burns on rabbits an inoculum of 10^4 to 10^5 c.f.u. *Staph. aureus* was necessary for 100 % colonization (Anthony & Wannamaker, 1967). Most experimental models of staphylococcal disease, however, bear little resemblance to the natural disease in man. It would seem that, even when protective gowns were used, secondary transfer of staphylococci by nurses' clothing to the air in the ward as well as directly to the patient could reach such levels as to afford a risk to the newly burned patient. Although the protective gown was insufficient for the conditions in this ward it did offer some protection to the patient. This protection may be sufficient for wards in which the amount of contamination is less than in a burns unit.

This investigation was supported by grants from the Swedish Delegation for Applied Medical Defence Research.

REFERENCES

ANTHONY, B. F. & WANNAMAKER, L. W. (1967). Bacterial interference in experimental burns. *Journal of Experimental Medicine* **125**, 319.

BERNARD, H. R., COLE, W. R. & GRAVENS, D. L. (1967). Reduction of iatrogenic bacterial contamination in operating rooms. *American Journal of Surgery* **165**, 609.

BETHUNE, D. W., BLOWERS, R., PARKER, M. & PASK, E. A. (1965). Dispersal of *Staphylococcus aureus* by patients and surgical staff. *Lancet* i, 480.

BLAIR, J. E. & WILLIAMS, R. E. O. (1961). Phage-typing of staphylococci. *Bulletin of the World Health Organization* **24**, 771.

BLOWERS, R. & WALLACE, K. R. (1955). Sterilisation of blankets with cetyl trimethylamine bromide. *Lancet* i, 1250.

BLOWERS, R. & MCCLUSKEY, M. (1965). Design of operating-room dress for surgeons. *Lancet* ii, 681.

CHARNLEY, J. & EFTEKHAR, N. (1968). Centre for Hip Surgery Wrightington Hospital, Internal Publication, no. 19.

DUGUID, J. P. & WALLACE, A. T. (1948). Air infection with dust liberated from clothing. *Lancet* ii, 845.

HALL, L. B. & HARTNETT, M. J. (1964). Measurement of the bacterial contamination on surfaces in hospitals. *Public Health Reports, Washington* **79**, 1021.

HAMBRAEUS, A. (1973). Dispersal and transfer of *Staph. aureus* in an isolation ward for burned patients. *Journal of Hygiene* **71**, 787.

HAMBRAEUS, A. & SANDERSON, H. F. (1972). Studies with an airborne particle tracer in an isolation ward for burned patients. *Journal of Hygiene* **70**, 299.

HARE, R. & THOMAS, C. G. A. (1956). The transmission of *Staphylococcus aureus*. *British Medical Journal* ii, 840.

LIDWELL, O. M. & TOWERS, A. G. (1972). Unidirectional ('Laminar') flow ventilation system for patient isolation. *Lancet* i, 347.

MICHAELSEN, G. S., HALBERT, M. M., SORENSON, S. D. & VESLEY, D. (1968). Development of an open isolation system for the care of low resistance hospital patients. Report from the National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare.

NICHOLES, P. S. (1970). Bacteria in laundered fabrics. *American Journal of Public Health* **60**, 2175.

ROBINTON, E. D. & MOOD, E. W. (1968). A study of bacterial contaminants of cloth and paper towels. *American Journal of Public Health* **58**, 1452.

DI SALVO, J. W. (1958). Deoxyribonuclease and coagulase activity of micrococci. *Medical Technicians Bulletin* **9**, 191.

SHINEFIELD, H. R., RIBBLE, J. C., BORIS, M. & EICHENWALD, H. F. (1963). Preliminary observations on artificial colonization of newborns. *American Journal of Diseases of Children* **105**, 146.

SPEERS, R., SHOOTER, R. A., GAYA, H., PATEL, N. & HEWITT, J. H. (1969). Contamination of nurses' uniforms with *Staphylococcus aureus*. *Lancet* ii, 233.

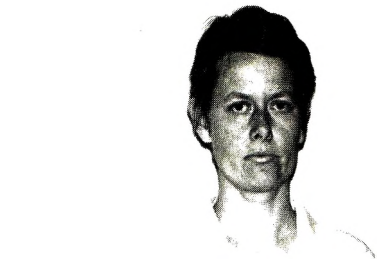
SYKES, G. (1970). The control of airborne contamination in sterile areas. *Aerobiology, Proceedings of the Third International Symposium* (ed. H. I. Silver). London and New York: Academic Press.

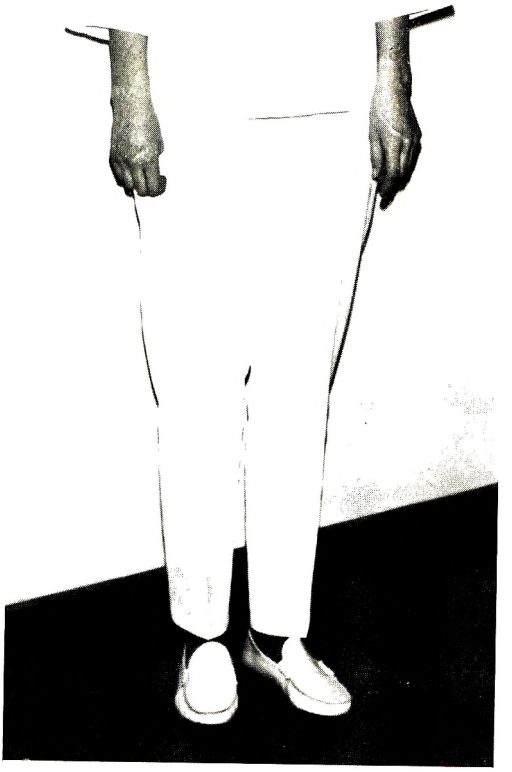
WILLIAMS, R. E. O. & SHOOTER, R. A. (1963). *Infections in Hospitals, Epidemiology and Control*. Blackwell Scientific Publications, Oxford.

EXPLANATION OF PLATES

Plate 1. Jacket and trousers used by ward staff.

Plate 2. Cotton surgical protective gown.





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J. Hyg., Camb. (1973), **71**, 815
Printed in Great Britain

Sources of contamination of cooked, ready-to-eat cured and uncured meats

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SUMMARY

Forty-five samples of unsliced, cooked, ready-to-eat meats on sale in retail premises and supermarkets were examined. Thirty-six (80%) had *Escherichia coli* I and 21 (47%) had coagulase positive staphylococci in numbers ranging from 1 to > 1000/100 cm.^{2}. Twenty-one samples contained *Clostridium* spp. in numbers from 1 to > 100/100 cm.^{2}. Of the 45 samples tested, 11 (factory-produced) and 7 (home-produced) were examined after cooking but before being offered for sale. Cooked hams were contaminated after handling in a factory, as were samples of canned corned beef after sale and exposure for 24 hr. Some sources of contamination were: (*a*) raw beef, (*b*) factory and shop surfaces and equipment, and (*c*) workers' hands. Curing brines used in retail shops and supermarkets to produce corned beef were a potent source of contamination. The effect of holding cooked meats at ambient temperature on their spoilage (22° C) and food-poisoning (37° C) microflora was demonstrated.

INTRODUCTION

Human food-poisoning is commonly associated with bacteria originating from animal sources; in most cases, infection is contracted indirectly by eating contaminated meat and meat products (Report, 1970). Such contamination may occur within the slaughterhouse (Walton, 1970) or in processing and handling before sale (Foster, 1972; Casman, McCoy & Brandly, 1963; Timoney, Kelly, Hannan & Reeves, 1970; Gilbert, 1969; Gilbert & Watson, 1971). The high incidence of bacterial food-poisoning in man (Morisetti, 1971) indicates that it is necessary to prevent contamination of meat and meat products in the food industry. In the case of cured products, the raw materials (beef and pork) and the curing brines used are potential sources of food-poisoning bacteria. For example, in the curing of beef, the cuts, usually silverside and brisket, are pumped with a brine and immersed in another brine for a number of days. The process may, or may not, be conducted under refrigeration. The cured beef, called corned beef, is usually displayed for sale immediately on removal from the curing tank. In the event of the brines and the corned beef containing potential food-poisoning organisms, there is

a real possibility of these being transferred to cooked cold meats where the latter are sold in close proximity to the former. This investigation was undertaken to determine whether such contamination could take place. Other sources of contamination of cooked, ready-to-eat meats were also investigated.

MATERIALS AND METHODS

Survey of registered food premises

In November 1971 a survey of registered food premises in County Dublin was carried out to examine the procedures used for the preparation and sale of pickled meat (beef and pork).

One hundred and twenty-three premises were registered under the Food Hygiene Regulations (1950) and these consisted of eighteen supermarkets with registered meat counters, and one hundred and five meat shops (pork and beef).

As a result of the survey it emerged that, in forty-two premises, there might be possible cross-contamination by potential food poisoning organisms from cured meat to cooked ready-to-eat products sold in the same premises.

Contamination seemed likely to occur through the handling and sale of both cured meat and cooked meat by the same workers using the same counters and weighing scales etc. The danger of cross contamination was not considered immediate in the remaining premises surveyed because cooked cold meats were sold by different workers using different scales and at different counters.

BACTERIOLOGICAL EXAMINATION

Retail shops

Cured meats

Forty-five samples of cooked meats on sale in 15 retail premises were examined. Of these, 18 were tested after cooking and before sale. Surfaces were swabbed by rubbing an area of 100 cm.^{2} with two cotton gauze pledgets (moistened with diluent) using a sterilized stainless steel template. The swabs were broken into a Universal bottle containing 20 ml. $\frac{1}{4}$ strength Ringer's solution + 0·1 % of added peptone. The bottles were shaken on a laboratory flask shaker for 5 min.

Escherichia coli I was enumerated by filtering 5 ml. of swab-rinse solution through an Oxoid membrane filter (Grade 0·45, 5 cm. diam.). The membrane was incubated on a pad soaked in resuscitation broth (Oxoid MM 20) for two hours at 37° C. and then transferred to a second pad soaked in MacConkey membrane broth (Oxoid MM 6*a*) in an aluminium airtight tin and incubated for 18 hr. at 44° C. in a watertight copper cylinder (Astell laboratory Services, Catford, London, S.E. 6) in a water bath at 44° C. ± 0·2° C.

Confirmation of *Escherichia coli* I was carried out using the Eijkman test (Mackie & McCartney, 1960). Typical lactose-fermenting colonies on the MacConkey broth impregnated membranes were picked off and purified on VRB agar (Oxoid CM 107). Tubes of brilliant green broth were inoculated and incubated in a water bath (44° ± 0·2° C.) for 18 hr.

The production of indole was tested by inoculating tubes of peptone water with

the cultures and incubating the tubes at 30° C. for 5 days. Indole was detected by adding Ehrlich's reagent.

Clostridium spp. were enumerated by filtering another 5 ml. portion of the swab rinse solution through the filter apparatus. The membrane was then placed on a plate of iron-sulphite agar (Oxoid CM 79) and overlaid with the same medium. The plates were incubated for 3 days anaerobically at 37° C. Coagulase positive staphylococci (CPS) were enumerated on the egg yolk – glycine – pyruvate – tellurite agar (EGPTA) of Baird-Parker (1962) by spreading 0·1 ml. serial dilutions on freshly prepared plates by the method of Davis & Bell (1959) and incubating the plates for 24–48 hr. at 37° C.

Fresh beef

Fresh beef (16 fore-ends and 16 hindquarters) was examined in the same way as the cured meats. Methods for the enumeration of *E. coli* I, *Clostridium* spp. and CPS were as described above.

Brines

Brines (31) from retail premises were collected in 120 ml. sterile plastic screw-capped jars and transported to the laboratory within 1 hr. A total count (25° C. for 5 days) was carried out by plating serial dilutions of the brine in 4 % (w/v) saline on plate count agar (PCA, Oxoid) + 4 % (w/v) of added NaCl. *E. coli* I and *Clostridium* spp. were enumerated by diluting 1 ml. of brine in 9 ml. of 4 % (w/v) saline + 0·1 % peptone and proceeding with the filtration technique as described earlier. Coagulase positive staphylococci were counted as described above.

Canned corned beef

One tin (3·2 kg.) of canned corned beef in each of six retail shops was opened aseptically and a 100 cm.^{2} area swabbed as described earlier. The butcher was asked to sell only half of each and retain the remaining portion for swabbing 24 hr. later. The procedures as described earlier were carried out to enumerate *E. coli* I, *Clostridium* spp. and CPS.

Equipment and surfaces

Shops' scales (11), slicing machines (10) and counter tops (6) were swabbed as described for the fresh beef samples and examined for *E. coli* I, *Clostridium* spp. and CPS.

Factory

Cooked hams

Ten hams, factory-cooked for 8 hr. at 158° F. (int. temp.) were opened aseptically and a 100 cm.^{2} area on each was swabbed. Each ham was allowed to follow the normal production pattern which consisted of peeling off jelly, trimming fat, cutting the larger hams into two, hand-packaging into new shrinkwrap plastic bags and drawing a vacuum. The packages were opened, then re-swabbed (100 cm.^{2}) to determine the degree of contamination during processing after cooking.

Equipment and surfaces

Equipment (aprons, knives, table tops) and the workers' hands were examined by swabbing, in each case a 100 cm.^{2} area.

Effect of holding cooked meats at constant temperatures

Nine samples of cooked, ready-to-eat meats (92 g. packets) were incubated at 20° C. ± 1° in a refrigerated incubator (Gallenkamp, London, E.C. 2) and at 2-hourly intervals total colony counts at 37° C. and 22° C. were made. Each sample was chopped up and mixed in a sterile jar. Ten grammes were weighed into 40 ml. sterile water, homogenized and 0·1 ml. serial dilutions in Ringer's solution plated on PCA containing 3% (w/v) of added NaCl. The plates were incubated for two days (37° C.) and 3 days (22° C.).

RESULTS

The degree of contamination found on the surface of cooked, ready-to-eat meats on sale in retail shops is shown in Table 1. *E. coli* I in excess of 1000 organisms/100 cm.^{2} was present in five samples of cooked ham and one sample of corned beef. Coagulase positive staphylococci were present in six cooked hams in similar numbers. Obviously this contamination resulted from the handling of these meats by personnel who also worked with other meats, e.g. raw meat and corned beef.

To determine the initial bacteriological condition of these meats, 18 were examined after cooking and before sale, i.e. before handling. The results are presented in Table 2. Three out of 11 factory-produced cooked hams and/or corned beef contained *E. coli* I in excess of 1000/100 cm^2 and two samples had coagulase positive staphylococci in excess of the same figure. Surprisingly only one out of seven samples of home-produced cooked meats and corned beef had numbers in excess of this figure, and the incidence of staphylococci on these samples was $< 1/\text{cm}^2$.

In order to establish the origin of the contamination of factory-produced cooked meats, 10 cooked hams were examined immediately after cooking and again after re-packaging. The results are presented in Table 3. As expected, the hams were free of the three 'indicator' organisms after cooking. However, the removal of the jelly, the trimming of fat, general handling and re-packaging, resulted in re-contamination. Thus, cooked hams can become contaminated even before they reach the retail level. Similarly, six tins (3·2 kg.) of corned beef which were aseptically opened proved to be sterile. However, after 24 hr. exposure they became contaminated.

In Table 4, some of the sources of contamination of cooked meats are tabulated. First, the raw beef bought in by the retailer (fore-ends and hindquarters) contained the 'indicator' organisms at various concentrations. The corned beef produced from this raw meat was similarly contaminated. Equipment (scales, slicing machines and counter tops) also contributed their quota of infection. In the factory, aprons, tables, knives and the hands of workers were found to be sources of *E. coli* I and staphylococci. *Clostridium* spp. were virtually absent from these surfaces.

Table 1. *Contaminated cooked, ready-to-eat meats on sale in 15 premises. Distribution of colony counts/100 cm.² of different organisms in various ranges*

Sample	No. tested	*Escherichia coli* I					*Clostridium* spp.				*Staphylococcus aureus*			
		0	1–10	11–100	101–1000	> 1000	0	1–10	11–100	> 100	0	1–100	101–1000	> 1000
Cooked hams	27	5	3	7	7	5	13	8	6	0	11	1	9	6
Corned beef ex tin	10	1	3	1	4	1	5	4	0	1	9	0	1	0
Other meats, e.g. brawn, hazlett, luncheon meat, roast pork and beef	8	3	3	1	1	0	6	1	1	0	4	3	1	0
Total	45	9	9	9	12	6	24	13	7	1	24	4	11	6
%	100	20·0	20·0	20·0	26·6	13·3	53·3	29·0	15·5	2·2	53·3	8·8	24·4	13·3

Table 2. *Contamination of meats in retail shops and supermarkets after cooking and before being offered for sale. Distribution of colony counts/100 cm.² of different organisms in various ranges*

Sample	No. tested	*Escherichia coli* I					*Clostridium* spp.			*Staphylococcus aureus*			
		0	1–10	11–100	101–1000	> 1000	0	1–10	11–100	0	1–100	101–1000	> 1000
(*a*) Factory-produced	11	3	1	3	1	3	7	2	2	6	0	3	2
(*b*) Home-produced	7	3	2	1	0	1	6	1	0	5	2	0	0
Total	18	6	3	4	1	4	13	3	2	11	2	3	2

(*a*) Nine were cooked hams and two were corned beef. Some of these meats were wrapped in greaseproof paper, the remainder were cellophane wrapped.

(*b*) Three were cooked hams, one was a sample of cooked pork, and three were corned beef. Some were unwrapped, the remainder were wrapped loosely in greaseproof paper.

Table 3. *Contamination of cooked hams during repackaging in factory and of canned corned beef on sale in retail shops. Distribution of colony counts/100 cm.² of different organisms in various ranges*

Sample	No. tested	*Escherichia coli* I*					*Clostridium* spp.				*Staphylococcus aureus*			
		0	< 10	10–100	101–1000	> 1000	0	< 10	10–100	> 100	0	< 100	> 100	> 1000
Factory-produced hams														
(1) After cooking	10	10	0	0	0	0	10	0	0	0	10	0	0	0
(2) After repackaging	10	2	2	6	0	0	9	0	1	0	6	0	3	1
Shop corned beef														
(1) Ex tin	6	6	0	0	0	0	6	0	0	0	6	0	0	0
(2) After 24 hr.	6	2	1	0	1	2	2	2	1	1	6	0	0	0

* Seven examined and six were positive (Eijkman test).

Table 4. *Sources of contamination of cooked meats in retail shops and a factory. Distribution of colony counts/100 cm.² of different organisms in various ranges*

Sample	No. tested	*Escherichia coli* I†					*Clostridium* spp.				*Staphylococcus aureus*			
		0	< 10	10–100	> 100	> 1000	0	< 10	10–100	> 100	0	< 100	> 100	> 1000
Retail shops														
1. Fresh beef														
(*a*) Fore-ends	16	4	4	2	6	0	5	5	4	2	10	3	3	0
(*b*) Hindquarters	16	2	6	4	4	0	4	9	2	1	12	2	2	0
2. Corned beef*	13	1	2	7	3	0	5	3	5	0	8	1	1	1
3. Equipment														
(*a*) Scales*	11	5	2	4	0	0	4	4	3	0	8	0	2	0
(*b*) Slicers*	10	9	1	0	0	0	7	3	0	0	8	0	0	0
(*c*) Counters	6	4	0	1	1	0	1	1	4	0	4	0	1	1
Factory														
1. Equipment														
(*a*) Aprons*	2	1	0	1	0	0	1	0	1	0	0	0	0	1
(*b*) Tables	2	0	0	2	0	0	2	0	0	0	1	0	1	0
(*c*) Knives	2	1	0	0	0	1	2	0	0	0	2	0	0	0
2. Hands of workers	4	0	0	4	0	0	4	0	0	0	0	0	3	1

* Two samples of corned beef, one set of scales, two slicing machines and one apron were not examined for *Staphylococcus aureus*.
† Eight examined, all were positive (Eijkman test).

Table 5. *Bacteriological analysis of curing (immersion) brines used in the production of corned beef*

No. of samples tested	No. of samples with total counts/ml.			No. of samples with *E. coli* I counts/ml.			No. of samples with *Clostridium* spp./ml.			No. of samples with staphylococcal counts/ml.*	
	< 10^6	10^6–10^7	> 10^7	< 10^2	10^2–10^3	> 10^3	< 10	10–10^2	> 10^2	< 10	⩾ 10
31	4	10	17	13	13	5	27	2	2	25	6

* Coagulase positive.

Table 6. *Effect of incubation at shop temperature of 20° C. on colony counts of cooked ready-to-eat cold meats*

	Counts/g. ($\times 10^4$) at							
	22° C. after (hr.)				37° C. after (hr.)			
Meats	0	2	4	6	0	2	4	6
Chicken/ham	3	1	2925	3075	2	1	2	15
Luncheon meat	1	93	4725	1950	2	4	9	4
Chopped ham	21	30	3000	190	1	1	3	7
Chicken/ham	276	300	500	535	1	7	5	4
Luncheon meat	2	3	3	5	62	91	75	200
Chopped ham	11	8	12	39	50	500	750	2828
Corned beef	21	42	47	25	83	127	100	108
Ham/beef	307	196	161	180	600	4000	3000	6875
Brawn	772	888	1120	668	600	1370	1408	1500

The bacteriological analysis of the curing brines used in retail shops and supermarkets for the curing (corning) of raw beef are shown in Table 5. Of 31 samples tested, 17 had total counts > 10^7 organisms/ml. The incidence of *E. coli* I, *Clostridium* spp. and coagulase positive staphylococci were also high; five samples had *E. coli* I > 10^3/ml.; 2 had *Clostridium* spp. > 10^2/ml. and 6 staphylococci > 10/ml.

The effect of exposure of cooked meats to shop temperature (20° C.) on the total counts at 22° C. and 37° C. is illustrated in Table 6. Irrespective of the initial bacterial count which varied at 22° C. from $0{\cdot}75 \times 10^4$ to $771{\cdot}5 \times 10^4$/g. and at 37° C. from $0{\cdot}45 \times 10^4$ to 600×10^4 g., with few exceptions all samples showed an actively increasing population at both temperatures over a 6 hr. period. Table 6 illustrates that where heavy initial contamination exists on cooked meats, and where these are displayed over a working day in, for example, the window of a retail shop or some other unrefrigerated environment, substantial multiplication takes place. The 37° C. count is particularly important.

DISCUSSION

Evidence is presented in this paper which links the curing of raw meat in retail premises with the contamination of cooked, cured, ready-to-eat meats. Personnel, equipment, utensils, raw materials (raw meat and brines) are potential sources of cross contamination. Although only 10 cooked hams were examined in a factory, each of these was found to be contaminated before reaching retail level. The situation is further complicated when these cooked meats are handled by shop assistants who cure and handle raw meat on the premises. It is not surprising that 86 % of all cases of food poisoning arise from made-up meat products, the vast majority of which are cooked meats (Simmons, 1972).

It is for reasons such as these that codes of hygiene require total and complete separation of cooked and raw meats.

The following precautions must be taken where cold meats are sold in the same premises as raw and cured (corned) beef:

(1) Complete separation of raw from cooked meats. This must include the provision of different surfaces and equipment for the handling, cutting, slicing,

weighing, display and sale of such meats (see Food Hygiene Codes of Practice, 1969).

(2) Washing (sanitizing) of hands and equipment before handling a cooked product. Preferably, different staff should be employed on the cooked meats section of the premises. Where possible, tongs, forks or other suitable instruments should be made available for handling a cooked product.

(3) Hold cooked meats under refrigeration, i.e. < 4·4° C. with free circulation of air. Since pathogenic bacteria grow between 4·4° and 48·8° C. (Elliott, 1972) cooked meats should not be permitted to be held in this range for long periods (see Table 6).

(4) Facilities for curing must be provided, preferably under refrigeration, in a separate room or an area remote from the shop proper. Immersion brines should be filtered or otherwise clarified before re-use.

(5) All persons engaged in the handling of meat, both in the factory and in the retail trade, must be provided with suitable protective clothing (protection for the meat, not the worker).

(6) Any person suffering from an infection of the stomach or intestine (vomiting, diarrhoea), septic cuts or boils must not handle meat (raw or cooked) until the condition has disappeared (see Food Hygiene Regulations, 1950).

We wish to record our appreciation to the Managers of the supermarkets and retail shops, without whose co-operation this work would not have been possible. To Miss C. Murphy and Mr B. Lynch our thanks for skilful technical assistance.

REFERENCES

BAIRD-PARKER, A. C. (1962). An improved diagnostic and selective medium for isolating coagulase positive staphylococci. *Journal of Applied Bacteriology* **25**, 12.

CASMAN, E. P., MCCOY, D. W. & BRANDLY, P. J. (1963). Staphylococcal growth and enterotoxin production in meat. *Applied Microbiology* **11**, 498.

DAVIS, J. G. & BELL, J. S. (1959). The 'drop' technique for colony counts in microbiology. *Laboratory Practice* **8**, 58.

ELLIOTT, R. P. (1972). The microbiology of equipment and processing. *Proceedings of the Meat Industries Research Conference* (March). American Meat Science Foundation, pp. 53–60.

FOOD HYGIENE REGULATIONS (1950). S. I. No. 205 of 1950. Stationery Office, Dublin.

FOOD HYGIENE CODES OF PRACTICE (1969). 8. *Hygiene in the Meat Trades.* Dept. of Health and Social Security, Ministry of Agriculture, Fisheries & Food (Welsh Office). H.M.S.O. pp. 14–16.

FOSTER, E. M. (1972). Spoilage and disease problems. *Proceedings of the Meat Industries Research Conference* (March). American Meat Science Foundation, pp. 61–72.

GILBERT, R. J. (1969). Cross-contamination by cooked-meat slicing machines and cleaning cloths. *Journal of Hygiene* **67**, 249.

GILBERT, R. J. & WATSON, H. M. (1971). Some laboratory experiments on various meat preparation surfaces with regard to surface contamination and cleaning. *Journal of Food Technology* **6**, 163.

MACKIE, T. J. & MCCARTNEY, J. E. (1960). *Handbook of Practical Bacteriology*, 10th ed. Ed. R. Cruickshank. London: E. & S. Livingstone.

MORISETTI, M. D. (1971). Public health aspects of food processing. *Process Biochemistry* **6**, 21.

REPORT (1970). The future structure of the National Health Service. Dept. of Health and Social Security, H.M.S.O. London.

SIMMONS, C. (1972). Hygiene in the meat trade. No. 8, cross contamination. *Meat Trades Journal* No. 4408, Nov. 23.

TIMONEY, J., KELLY, W. R., HANNAN, J. & REEVES, D. (1970). A study of salmonella contamination in some Dublin poultry processing plants. *Veterinary Record* **87**, 158.

WALTON, J. R. (1970). Contamination of meat carcasses by antibiotic-resistant coliform bacteria. *Lancet* ii, 561.

J. Hyg., Camb. (1973), **71**, 825
Printed in Great Britain

A study of immunity to rubella in villages in the Fiji Islands using the haemagglutination inhibition test

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(*Received* 24 *April* 1973)

SUMMARY

In the villages of Fiji, apart from Viti Levu, rubella is a disease occurring solely in widely spaced epidemics. Some villages may not be infected for over 20 years and will then contain substantial numbers of susceptible women of child-bearing age.

Evidence is produced that haemagglutination-inhibiting (H.I.) antibody to rubella is very long lasting in Fijians. The infectivity of the virus is discussed and it is suggested that, on the average, 50% of susceptibles are infected in a Fijian village during a rubella epidemic, but there are large variations.

INTRODUCTION

Fiji consists of a group of over 300 islands, at least 100 of which are inhabited. They are situated between longitudes 177° E. and 178° W. and latitudes 16° and 19° S., with a few outlying islands extending beyond these limits. The main island of Viti Levu has a land area of 4011 sq. miles. It has a road navigable by ordinary cars right round the island and several other fair roads. It is on the air routes between Australasia and North America and receives large numbers of tourists. The other islands have not got such good communications and some of the smaller islands are very isolated. The total population was approximately 477,000 at the 1966 census.

Because of the great variations among villages of the Fiji group in their sizes and degrees of contact with each other and outside communities the question arose whether the epidemiology of rubella would be relatively uniform amongst the villages and whether it would be comparable to that shown by surveys in other regions of the world.

Areas studied

The map (Fig. 1) shows the islands on which the villages studied were situated and the name and locations of the study villages on Kadavu and Vanua Levu.

Five villages in the Lau group were studied. These isolated islands are the farthest to the east. Nasau is on the island of Moce and Dravuwalu on Totoya.

Lomaiviti includes the island of Ovalau and smaller offshore islands. Our studies were made on two villages on Gau, Lawaki on Nairai and Yavu on Batiki.

The villages on Kadavu were all in locations which can only be reached by boat.

Most of those on Vanua Levu were also only to be reached by boat or on foot, but Wailevu was close to the main town Labasa and in easy communication with it and some others were accessible by road. Kadavu has an area of 159 sq. miles and Vanua Levu 2,137 sq. miles.

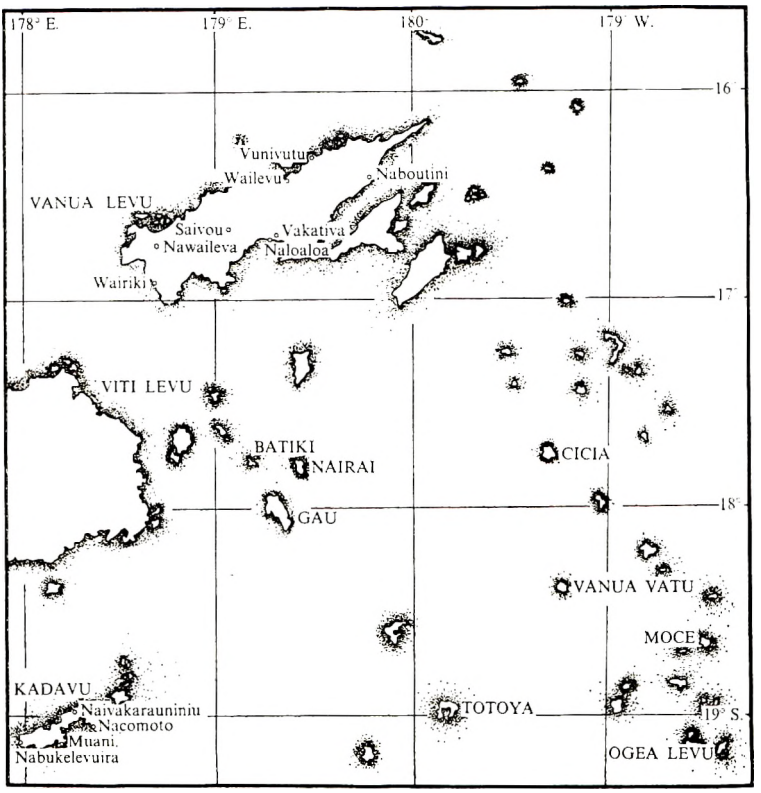

Fig. 1. Part of Fiji Island group showing islands studied.

MATERIALS AND METHODS

Serum specimens

Venous blood specimens from females in all age groups were separated in the field and transported back to Suva on wet ice. They were then stored at $-20°$ C. until used.

Antigens

Rubella haemagglutinating and control antigens were obtained from Microbiological Associates, Bethesda, Maryland.

Red blood cells

Fresh chick red blood cells were obtained for each test series. The cells were triple washed and suspended at the appropriate concentration.

Table 1. *Summary of results of testing sera from females for H.I. antibodies to Rubella virus*

Island group	Village	No. tested	No. +ve	% +ve	Age in years of youngest +ve
Lau	Ogea	57	20	35	20
	Cicia	55	25	45·5	12
	Nasau	131	61	46·6	9
	Dravuwalu	73	51	69·9	10
	Vanua Vatu	89	51	57·2	9
Lomaiviti	Navukailagi	49	24	49	9
	Lawaki	31	18	58	16
	Yavu	34	10	29·4	20
	Nukuloa	57	13	22·8	21
Kadavu	Nabukelevuira	79	43	54·4	4
	Nacomoto	69	40	58	11
	Muani	76	44	58	10
	Naivakarauniniu	32	19	59·4	11
Vanua Levu	Saivou	31	25	80·6	8
	Vunivutu	73	52	71·2	7
	Naboutini	39	21	53·8	11
	Naloaloa	30	22	73·3	9
	Wairiki	23	8	34·8	28
	Wailevu	78	51	65·4	8
	Nawailevu	41	23	56	8
	Vakativa	27	19	70·3	9

Test

The tests were carried out in Microtitre plates as a screening test at a serum dilution of 1/4 following the technique described by Plotkin (1969).

RESULTS

The results are summarized in Tables 1 and 2 and more details of individual villages in the Lau group are shown in Fig. 2. Only in one village was any serum from a child under 5 years old positive. At Nabukelevuira on Kadavu one 4-year-old was positive and since a 5-year-old was also positive it seems likely that rubella did occur in this village in 1965 or a little before. In none of the other three Kadavu villages studied were there any positives under the age of 10 years although a total of 41 sera from children under the age of 10 was tested from them.

Four villages had no positive sera from persons under the age of 20. In Ogea in the Lau group 35 sera from individuals below that age were tested.

Over the whole survey 90 % of the 40–49 age group were positive, 100 % of the 50–59 and 95 % of those 60 years old or more.

DISCUSSION

The results of this survey are substantially different from those which have been published previously. The extensive WHO collaborative study (Rawls *et al.* 1967) shows that in large centres in the U.S.A., continental South America, Europe and

Table 2. *H.I. antibodies to Rubella in four groups in the Fiji Islands: numbers of individuals with antibody at different ages*

Ages...	0–4		5–9		10–14		15–19		20–9		30–9		40–9		50–9		60+		Totals		% +ve
Island group	No. tested	+	No. tested	+	No. tested	+	No. tested	+	No. tested	+	No. tested	+	No. tested	+	No. tested	+	No. tested	+	No. tested	+	—
Lomaiviti	7	0	44	1	18	2	21	4	19	8	22	15	20	17	10	10	10	8	171	65	38
Lau	34	0	74	2	58	21	57	31	52	41	54	40	32	29	21	21	23	23	405	208	51
Kadavu	15	1	48	2	35	17	20	15	42	26	32	26	24	20	21	21	19	18	256	146	57
Vanua Levu	29	0	57	12	42	20	33	24	50	41	63	57	32	31	20	20	16	16	342	221	65
Totals	85	1	223	17	153	60	131	74	163	116	171	138	108	97	72	72	68	65	—	—	—
Percentage +ve	1·2		7·6		39·2		56·4		71		81		90		100		95·4		—		—

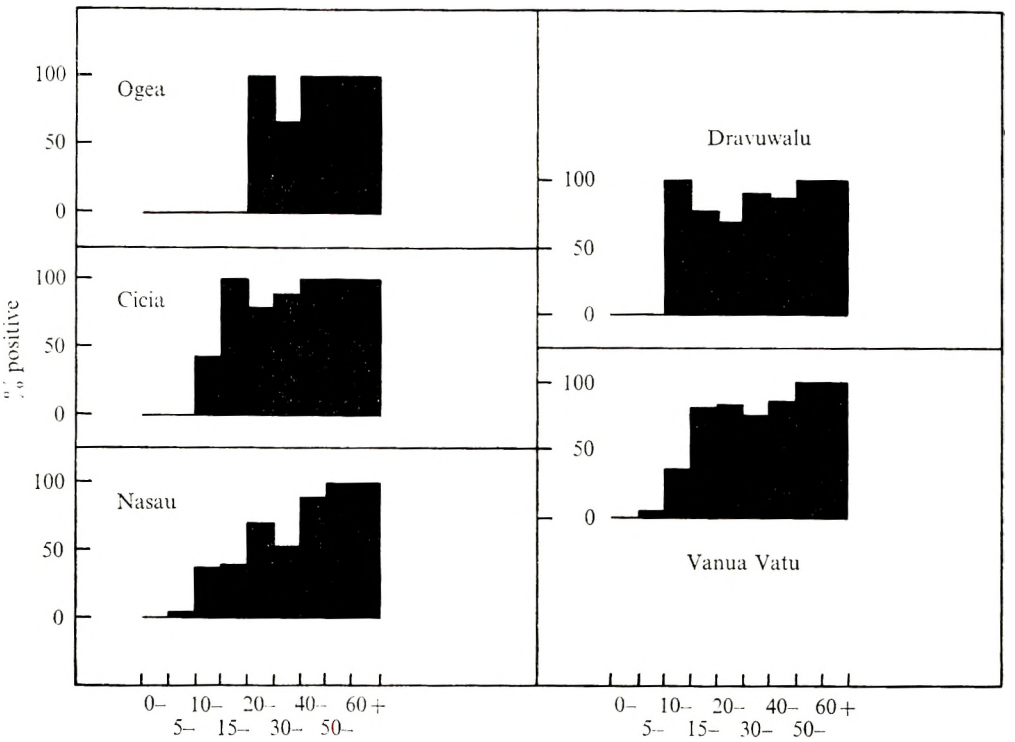

Fig. 2. H.I. antibodies of Rubella virus in various villages of the Lau Islands. Percentage of females with antibody in different age groups.

Australia 50 % or more of children had antibodies against rubella virus by the age of 8 years and the proportion of women positive by the age of 17 was near 80 %. In Japan the figures for children were similar, but the percentage of positive adults was lower. This study showed lower positive rates in Jamaica and Trinidad.

A later collaborative study (Dowdle *et al.* 1970) confirmed these results for continental South America and the Carribean and showed that, while in most countries there was little difference between urban and rural populations, in Peru, Jamaica, Panama and Trinidad the percentage positive in the rural 5–9 age group varied between 25 and 33 %. A further study (Golubjatnikov, Elrea & Leppla, 1971) found that Mexican children showed 76 % of positives by the age of 7 and 100 % by 13. In Paraguay only 17 % were positive at the age of 7 and 80 % of positives was reached only at 15 years.

The delay in development of antibodies in the rural areas of some countries suggests that rubella is not so infectious as measles. Our results show clearly that in the villages we have studied rubella is an epidemic disease which, despite its ability to survive in the presence of neutralizing antibody at least in congenitally infected infants, is unable to remain endemic under conditions found in Fiji.

Epidemics are infrequent and apparently fail to spread uniformly throughout the villages on the larger islands as well as to all the small islands. An interesting example is Gau island in Lomaiviti where Navukailagi had an epidemic approxi-

Table 3. *H.I. antibodies to Rubella virus with suggested number of exposures*

	Exposures								
	None			One			> 1		
Island group	Total	+ve	%+ve	Total	+ve	%+ve	Total	+ve	%+ve
Lau	115	0	0	108	48	44·4	182	160	88
Lomaiviti	72	0	0	52	22	42·3	47	43	91·5
Kadavu	47	0	0	90	40	44·4	119	106	89
Vanua Levu	90	3	3·3	88	58	65·9	164	160	97·6
Totals	324	3	0·9	338	168	49·7	512	469	91·6

mately 9 years before the survey was undertaken; while Nukuloa on the other side of this small island and through which most communication with larger centres takes place had been unaffected for over 20 years.

Figure 2 indicates that there are great differences in the proportion of susceptibles infected in different epidemics and different villages. However, an attempt was made to assess the average proportion of susceptibles infected during a single exposure under the conditions in the Fiji group and assuming that few infected persons, if any, lose antibody detected by the H.I. test. This assumption is supported by the observation, typical amongst others, that in Ogea where no infection had occurred for 20 years, 22 out of 24 persons tested, aged 20 years or more, had antibody; and the finding that all 72 sera tested from women between the ages of 50 and 59 and 65 of 68 from those 60 years old or more were positive. However, Freestone, Rowlands & Prydie (1972) have found a relatively poor correlation between rubella antibodies and a history of rubella except when the attack was recent, and favour the theory that this is due to loss of antibody, but in view of the difficulty of making a clinical diagnosis of infection with rubella virus and the difficulty many patients have in recalling minor illnesses, other explanations are possible.

If our assumptions are correct then our data indicate that the average percentage of susceptibles infected in a rubella epidemic is about 50 %, although the variation from village to village is very large (Table 3 and Fig. 2).

Finally this study has revealed the existence of large numbers of susceptible women of child-bearing age and suggests that in Fiji and in other areas with a similar distribution of population serious consideration should be given to developing a rubella vaccination programme designed to protect this group as far as possible.

This work was assisted by the New Zealand Council for Medical Research. It is a pleasure to acknowledge the help of Dr J. U. Mataika and the staff of the filariasis survey for the help in the collection of the specimens.

REFERENCES

DOWDLE, W. R., FERREIRA, W., DE SALLES GOMES, L. F., KING, D., KOURANY, M., MADALENGOITIA, J., PEARSON, E., SWANSTON, W. H., TOSI, H. S. & VILCHES, A. M. (1970). W.H.O. collaborative study on the sero-epidemiology of rubella in the Caribbean and Middle and South American populations in 1968. *Bulletin of the World Health Organization* **42**, 419.

FREESTONE, D. S., ROWLANDS, D. F. & PRYDIE, T. (1972). History of infection and immunity to rubella. *American Journal of Epidemiology* **96**, 70.

GOLUBJATNIKOV, R., ELREA, W. R. & LEPPLA, L. (1971). Measles and rubella H.I. antibody patterns in Mexican and Paraguayan children. *American Journal of Tropical Medicine and Hygiene* **20**, 958–63.

PLOTKIN, S. A. (1969). Rubella virus. In *Diagnostic procedures for viral and rickettsial diseases*, 4th edn. (Eds. E. H. Lennette and N. J. Schmidt), pp. 364–413. New York, American Public Health Association.

RAWLS, W. E., MELNICK, J. L., BRADSTREET, C. M. P., BAILEY, M., FERRIS, A. A., LEHMANN, N. I., NAGLER, F. P., FURSZ, J., KONO, R., OHTAWARA, M., HALONEN, P., STEWART, J., RYAN, J. M., STRAUSS, J., ZDRAZILEK, J., LEERHOY, J., VON MAGNUS, H., SOHIER, R. & FERREIRA, W. (1967). W.H.O. collaborative study on the sero-epidemiology of rubella. *Bulletin of the World Health Organization* **37**, 79.

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THE
JOURNAL OF HYGIENE

THE
JOURNAL OF HYGIENE

(Founded in 1901 by G. H. F. Nuttall, F.R.S.)

VOLUME LXXI. 1973

CAMBRIDGE
AT THE UNIVERSITY PRESS
1973

PUBLISHED BY
THE SYNDICS OF THE CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London NW1 2DB
American Branch: 32 East 57th Street, New York, N.Y. 10022

Printed in Great Britain at the University Printing House, Cambridge

Contents

No. 1 (March 1973)

No. 3 (SEPTEMBER 1973)

The Journal of Hygiene Volume 71, No. 4 December 1973

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Printed in Great Britain at the University Printing House, Cambridge