Vol. 68, No. 4 December 1970

THE JOURNAL OF HYGIENE

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

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CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London, N.W.1

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

Subscription per volume £7 *net* ($23.00) (4 parts)

Single parts 40*s. net* ($6.50)

The Journal of Hygiene

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The Journal of Hygiene is published quarterly.

Single parts 40*s*. net ($6.50 in the U.S.A.) plus postage.

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Cambridge University Press

Bentley House, 200 Euston Road, London, N.W. 1

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

J. Hyg., Camb. (1970), **68**, 511
Printed in Great Britain

Sampling rabbit pox aerosols of natural origin

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(*Received* 16 *April* 1970)

SUMMARY

Natural aerosols of rabbit pox virus produced by groups of infected rabbits were sampled with a slit and Andersen sampler using an adhesive surface sampling technique (Thomas, 1970). The higher rate of successful recoveries of airborne virus compared with a previous report is believed to be due to the much larger volume of air sampled by this technique and to the processing of the whole of the sample collected by the direct addition of a cell suspension to demonstrate the presence of viable virus.

INTRODUCTION

Natural aerosols of the pox viruses have been sampled with varying degrees of success (Meiklejohn *et al.* 1961; Downie *et al.* 1965; Westwood, Boulter, Bowen & Maber, 1966). Sampling methods used included settle plates, cotton wool filters, sieve samplers, impingers and an electrostatic precipitator (Morris, Darlow, Peel & Wright, 1961). The opportunity arose of sampling natural rabbit pox aerosols under the same conditions as those reported by Westwood *et al.* (1966) with groups of animals infected with rabbit pox. Air samples were taken using an adhesive surface sampling method which had proved effective with artificial aerosols of vaccinia virus (Thomas, 1970).

MATERIALS AND METHODS

Samplers. Slit sampler (Casella) and an automated version. Andersen sampler (Andersen, 1958).

Cell culture. HeLa cells (Appleyard & Westwood, 1964). Monolayers prepared from 7–8 × 10^6 cells, incubated at 37° C. under 5 % CO_2 for 2 days.

Nutrient medium. '199', with neomycin (70 units/ml.), amphotericin B (0·0025 mg./ml.), calf serum 5 %.

Rabbit pox virus. Utrecht strain.

Rabbits. Mixed sexes, about 2·5 kg.

Infection method. Respiratory route (Westwood *et al.* 1966).

Sampling methods. Petri dishes were coated with 0·2 ml. of either 10 % calf serum, 10 % bovine serum albumin or a mixture of equal volumes of saturated sucrose solution and glycerol with 0·1 % of 10 % bovine serum albumin (S.G.B. mixture, Thomas, 1970). Sampling periods of 1 hr. were used. A HeLa cell suspension was then added to each plate to form a monolayer in direct contact with the aerosol particles that had been collected. Plaque counts were taken after 2 days.

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RESULTS

Three experiments were carried out with different groups of rabbits.

Experiment 1

Air samples were taken with a slit sampler from a room containing five infected rabbits via a 1 in. diameter tube passing through a hole in a door. Ten per cent calf serum was used as the adhesive sampling surface and six consecutive 1-hr. samples were taken on the 5th day after infection. Only one animal had a slight nasal discharge at this time and no other signs of infection appeared. The plaque counts obtained from the samples are given in Table 1.

Table 1. *Expt* 1, *Sampling natural rabbit pox aerosol using* 10% *calf serum as an adhesive surface*

Sample no.	Plaque counts	Vol. of air/ sample (cu.ft.)
1	2	60
2	5	
3	1 (p.m.)	
4	7	
5	10	
6	3	

p.m., Poor monolayer.

Experiment 2

Ten per cent bovine serum albumin was used to coat the plates. Sampling was carried out as described in the first experiment, the room containing 30 infected rabbits. Groups of consecutive 1-hr. samples were taken over a period of 7 days commencing on the 4th day after infection. Plaque counts are given in Table 2. An Andersen sampler was used for one sample on the 8th day after infection. Glass disks coated with 10% bovine serum albumin replaced the agar in the sampling dishes. The disks were of the right thickness to establish the correct distance between the under surface of the orifice plates and the collecting surfaces. Table 3 gives the plaque counts obtained with the Andersen sampler.

Experiment 3

In this investigation samplers were placed inside the room holding the rabbits on the 8th day after infection. There were 33 animals, 12 with rashes and seven had discharges. The slit sampler and the automated slit sampler were used with the sample plates coated with the S.G.B. mixture. Five consecutive 1-hr. samples were taken in parallel with the two samplers and the resulting plaque counts are given in Table 4.

DISCUSSION

The same room was used for all three experiments and the general conditions, temperature, humidity, ventilation, feeding and cleaning routines, sampling periods, etc., were similar. Approximately six air changes an hour took place

continuously causing fairly rapid dilution of the aerosols produced by the animals. The cubic capacity of the room was approximately 4500 cu.ft.

In the first experiment 10 % calf serum was used to coat the sampling dishes. It is likely that efficient sampling occurred only during the first 20 min. each plate

Table 2. *Expt* 2, *Sampling natural rabbit pox aerosol using* 10 % *bovine serum albumin as an adhesive surface*

				Number of rabbits			
					With		
Sample no.	Plaque count	Vol. of air/sample (cu.ft.)	Days after infection	Live	Discharges	Rash	Dead
1	0						
2	0						
3	0						
4	0	60	4	30	0	0	0
5	0						
6	0						
7	6						
8	0	60	5	30	2	5	0
9	1						
10	1						
11	1						
12	1	60	7	30	5	12	0
13	1						
14	3						
15	2						
16	7						
17A	86						
18	2	60	8	29	10	15	1
19	2						
20 p.m.	0						
21	25	60	—	—	—	—	—
22	12						
23	74	120	9	27	9	15	2
24	1						
25	1	60	—	—	—	—	—
26	1						
27	13						
28 p.m.	0						
29	81						
30	13	60	10	25	10	18	2
31 p.m.	0						
32	4						
33	1						

Sample 17A, Andersen sampler—plaque count is total of plaques on all stages of the sampler. p.m., Poor monolayer.

was used since the calf serum dries out to give a smooth non-adhesive surface. Five animals were used in this first experiment and of these only one had evidence of infection so that the amount of viable airborne virus produced was probably small. Despite these limiting factors all the samples were positive.

The bovine serum albumin used in the second experiment behaves similarly to the calf serum. It was considered to have a marginal advantage in not containing the inhibitory factors sometimes present in calf serum. The single sample taken on the 5th day after infection was negative despite the presence of two animals with discharges. Both were on the far side of the room away from the sampling orifice in the door, while in the first experiment the cages were placed as close as possible to the mouth of the sampling tube.

Table 3. *Expt* 2, *Sampling natural rabbit pox aerosol using an Andersen sampler and* 10 % *bovine serum albumin as an adhesive surface*

Stage no.	Plaque count	Distribution (%)	Total vol. of air sampled (cu.ft.)
1	25	29	
2	17	20	
3	24	28	60
4	15	17	
5	4	5	
6	1	1	

Table 4. *Expt* 3, *Sampling of rabbit pox aerosol with a standard and an automated slit sampler, using S.G.B. mixture as the sampling medium*

Sample no.	Plaque counts: Slit sampler	Plaque counts: Automated sampler	Vol. of air/sample (cu.ft.)
1	17	6	
2	24	14	
3	16	5	60
4	6	28	
5	16	17	
Totals	79	70	300

The samples taken on the 7th, 8th and 9th days after infection showed increasing total plaque counts in keeping with the progress of the disease in the animals. Where virus recoveries were higher in the mornings this was probably due to feeding and cleaning.

The results of previous investigations with the adhesive surface sampling technique (Thomas, 1970) suggest that the individual plaques in the samples taken with the slit and Andersen samplers represent separate aerosol particles carrying virus. In the Andersen samples taken on the 8th day viable virus was mainly carried on the larger particles collected in the upper four stages of the sampler, i.e. $> 2{\cdot}5\,\mu$.

For the third experiment the plates were coated with the S.G.B. mixture which remained fully adhesive throughout the sampling period. The samples were taken in the afternoon of the 8th day after infection when feeding and cleaning had been completed. This had been a period of low plaque counts in the second

experiment, and the higher counts obtained during this time in the third experiment are due in large part to sampling inside the room and the superior collection efficiency of the S.G.B. mixture. It remained adhesive for the 60 min. exposure in the sampler while the calf serum and bovine serum albumin dried out to give a smooth, non-adhesive surface. Impaction on such a surface has been shown to result in loss of small particles due to break up of aggregates or whole particles on impact (Davies, Aylward & Lacey, 1951). Many of the fragments would not be retained by a non-adhesive surface but be swept on by the air stream.

Table 4 shows considerable variation in the plaque counts for simultaneous samples taken by the two slit samplers. However, the total counts for the whole sampling period were very close, 79 and 70. The automated slit sampler was a prototype version which enabled samples to be taken at chosen intervals for pre-set periods at 1 cu.ft. of air/minute. Its sampling efficiency was closely similar to the standard slit sampler. In this experiment the machine was set to take five 1-hr. samples consecutively while the standard sampler was serviced manually at 1-hr. intervals.

When poor monolayer formation occurs, and it is not possible to make a reliable plaque count, the contents of the sample plate can be subcultured onto another monolayer and viable virus detected. The plaque counts in these circumstances, however, would not be related to particles collected.

Westwood *et al.* (1966) reported experiments in air sampling for rabbit pox virus which were undertaken in the same rooms and under conditions similar to those in the first and second investigations reported above. Sampling was carried out with raised glass impingers (May & Harper, 1957) and an electrostatic precipitator (Morris *et al.* 1961). Morris has shown (unpublished) that the impinger and the precipitator have similar sampling efficiencies in the range down to 1 μ size particles, but the precipitator has a superior collection efficiency for submicron particles. A series of samples were taken over the period between the 3rd and 12th days after infection inclusive. Most of the samples were obtained with the precipitator but some parallel samples were taken with impingers. The latter were operated for 15 min. each sample at 10 l./min., while the precipitator was run at the same rate for 30 min./sample. Airborne virus was recovered only with the electrostatic precipitator on the 6th and 7th days after infection. The air sampling results reported by Westwood *et al.* (1966) were a summary of seven experiments in which the numbers of animals used varied, but in all cases exceeded the numbers used in each of the rabbit pox experiments reported above. Onset of infection and the ensuing progress of the disease in the various groups of animals used was closely similar to that observed in the experiments described above but more developed discharges.

The rabbit pox aerosol arises from the infected discharges produced by the animals. Westwood *et al.* (1966) examined these discharges and found that the virus content reached maximum levels on the 6th and 7th days after infection. This corresponded with their successful aerosol samples. But their findings also showed that these discharges continued to have a high titre of virus up to the

12th day. However, they obtained no positive air samples on the 8th to the 12th days.

The lack of success in the air sampling carried out by Westwood *et al.* (1966) was due to a number of factors. Most important was the relatively small volume of air taken in each sample and with only representative quantities of the sampling liquid inoculated into hen's eggs some virus might have been missed in this way. In addition the sampling characteristics of the impingers and the electrostatic precipitator used tended to discriminate against collection of the larger particles from a heterogeneous aerosol. The results (Table 3) of the Andersen sampler indicated a preponderance of particles larger than 5 μ carrying viable virus.

Brachman *et al.* (1964) recommended that data obtained with any specialized sampler should be correlated with results obtained with a standard impinger reference sampler. In a previous report (Thomas, 1970) comparisons were made between the impinger and the adhesive surface sampling technique using artificial virus aerosols. However, in the investigations reported above, it was decided not to use an impinger in view of the failure to recover viable airborne virus reported by Westwood *et al.* (1966) and its known particle discrimination behaviour. In addition the adhesive surface sampling technique had been developed specifically to obtain long sampling periods while the impinger sampler has a relatively short sampling period. Brachman *et al.* (1964) also recommended comparison with an Andersen sampler. In this case the adhesive surface sampling method is directly applicable to the Andersen sampler and has been shown to work successfully with this sampler both with artificial virus aerosols (Thomas, 1970) and with a natural virus aerosol as reported above.

The successful recovery of airborne virus in the three experiments described in this report is a consequence essentially of the large volumes of air sampled, coupled with the wide particle range collected by the samplers and the fact that the whole of each sample taken was processed to demonstrate viable airborne virus.

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J. Hyg., Camb. (1970), **68**, 519
With 1 *plate*
Printed in Great Britain

Differentiation of the serological response to *Yersinia enterocolitica* and *Brucella abortus* in cattle

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(*Received* 30 *April* 1970)

SUMMARY

The serological responses of cattle to inoculation with *Brucella abortus* and *Yersinia enterocolitica* type IX were compared. Complete cross-reactions were found in serum agglutination, antiglobulin, complement fixation and Rose Bengal plate tests. The cross-reaction between *Br. abortus* and *Y. enterocolitica* IX was confirmed by immunodiffusion tests. Although antibodies specific for each organism could also be detected by immunodiffusion tests with high titre rabbit or bovine sera, these tests were insufficiently sensitive for routine diagnostic use.

A quantitative Rose Bengal plate test, using Rose Bengal stained *Br. abortus* and *Y. enterocolitica* IX, was developed which enabled the antibody responses to the two organisms to be differentiated. The specificity of this test was confirmed by cross-absorption experiments and its sensitivity was sufficient to permit evaluation of all bovine sera giving positive reactions to the serum agglutination test.

INTRODUCTION

Serological cross-reactions between organisms of the genus *Brucella* and those of other genera, notably *Pasteurella*, *Francisella* and *Vibrio*, have been reported in the past (Mallmann, 1930; Morse, Ristic, Robertstad & Schneider, 1953). Most of these reactions have been marked by quantitatively lower titres to the heterologous organism. However, recently Ahvonen, Jansson & Aho (1969) demonstrated strong serological cross-reactions between *Brucella abortus* and strains of *Yersinia enterocolitica* (Frederiksen, 1964) of serological type IX. Ahvonen & Sievers (1969) also observed the development of high titres of brucella agglutinins in the sera of patients infected with *Y. enterocolitica* IX. These observations were made in Finland, a country from which bovine brucellosis has been eradicated (Huhtala, 1963).

Y. enterocolitica, previously known as *Pasteurella* X or *Bacterium enterocoliticum* (Schleifstein & Coleman, 1943), is distinct from brucella in a number of morphological, cultural and biochemical characteristics (Mollaret & Chevalier, 1964). The organism is widely distributed and has been isolated from man and other animals, including hares, chinchillas, pigs, dogs, cattle and the bush-baby (Dickinson & Mocquot, 1961; Becht, 1962; Daniels, 1963; Mollaret & Lucas, 1965; Nilèhn, 1967; Goyon, 1969; Mair, Schubert & Harbourne, 1970). Most isolations

appear to have been reported from Northern Europe and North America. Hitherto the organism has not been recovered from cattle in Great Britain. However, the serological relationship of brucellas to other organisms is obviously of considerable significance in the diagnosis of human and bovine brucellosis, particularly in relation to brucella eradication schemes. It was the object of the present work to examine the cross-reactions between *Y. enterocolitica* IX and brucellas in the various serological tests used in the diagnosis of bovine brucellosis, and if possible to devise a means of differentiating between the serological responses to the two organisms.

MATERIALS AND METHODS

Bacterial strains

Yersinia *strains.* A strain of *Yersinia enterocolitica* type IX was kindly donated by Dr P. Ahvonen, of the Municipal Bacteriological Laboratory, Helskini, Finland, as a freeze-dried culture. On examination it conformed to the biochemical and cultural characters described by Mollaret & Chevalier (1964) and in these respects was identical with another strain of *Y. enterocolitica* (N.C.T.C. 10,461) obtained from the National Collection of Type Cultures, Colindale. Serologically, the Finnish strain was grouped as *Y. enterocolitica* type IX. It did not auto-agglutinate in 0·85 % saline nor in acriflavine solutions (Alessandrini & Sabatucci, 1931) and was apparently a smooth strain.

Brucella *strains. Br. abortus* strain 99 was used for preparation of the antigens used throughout most of this work. Animal inoculations were performed with *Br. abortus* strain 19 and some serological tests were done with antigens prepared from *Br. melitensis* strain 16M, *Br. neotomae* 5K33 and *Br. suis* strain 1330. All brucella strains were from the *Brucella* type culture collection maintained at this laboratory.

Preparation of antigens

Br. abortus strains 19 and 99 were grown in continuous culture and harvested according to Boyce & Edgar (1966).

Other brucella strains and *Y. enterocolitica* IX were grown on serum–dextrose agar in Roux flasks. Brucella cultures were grown at 37° C. for 3 days and *Y. enterocolitica* IX cultures at 22° C. for 18 hr. Organisms were harvested in 0·85 % saline, killed by exposure to 0·4 % β-propiolactone (LoGrippo & Hartman, 1955) and freed of growth medium by repeated washing with saline.

Standard *Br. abortus* agglutination suspensions were prepared according to WHO monograph no. 19 (1953) and were standardized to give 50 % agglutination with 1/500 dilution of the International Standard *Br. abortus* antiserum. *Y. enterocolitica* IX suspensions similarly prepared, were nephelometrically standardized to the same cell density as the standard *Br. abortus* suspensions. OH-antigen suspensions of *Y. enterocolitica* IX were also prepared as described by Winblad, Niléhn & Sternby (1966). O antigen suspensions were prepared from cultures grown at 37° C. and heated at 100° C. for 15 min.

Rose Bengal plate test (RBPT) antigens

Standardized suspensions of *Br. abortus* strain 99 stained with Rose Bengal and buffered at pH 3·65 were prepared according to U.S.D.A., National Animal Disease Laboratory, Diagnostic Reagents Manual 65 C (1965). Similarly stained and buffered suspensions of *Y. enterocolitica* type IX were prepared and standardized to the same packed cell volume as the brucella suspensions.

Soluble antigens

Washed suspensions of *Br. abortus* strain 99 or *Y. enterocolitica* type IX were suspended in 0·5 M-KCl containing 0·1 % cysteine hydrochloride and subjected to ultrasonic vibrations for two periods of 10 min. in a Soniprobe ultrasonic disintegrator (Dawe Instruments Ltd. London). Cell wall debris was removed by centrifugation at 20,000 *g* for 2 hr. and the supernatant dialysed against 0·85 % saline for 24 hr. The resulting solutions were adjusted to the same dry-weight concentration and used as antigens in the complement fixation (CF) test. Soluble antigens for immunodiffusion tests were produced by a similar process except that centrifugation of the disrupted organisms was at 20,000 *g* for 30 min.

Antisera used

Bovine antisera to *Br. abortus* were prepared by inoculation of two 18-month-old bullocks (B 1 and B 2) with single doses of $1 \cdot 8 \times 10^{11}$ viable *Br. abortus* strain 19 organisms by the subcutaneous route.

In addition anti-brucella sera were obtained from female calves vaccinated at between 3 and 6 months of age with the standard dose of *Br. abortus* strain 19 vaccine prepared at this laboratory. In each case blood samples were taken before inoculation and at weekly intervals after.

Bovine antisera to *Y. enterocolitica* type IX were prepared by inoculation of four 18-month-old bullocks (Y 1, Y 2, Y 3 and Y 4) by the subcutaneous route with 2 ml. volumes of a washed suspension of *Y. enterocolitica* IX in Ringer's solution. This suspension contained 3×10^{10} viable organisms per ml. as determined by the method of Miles & Misra (1938). Blood samples were taken at weekly intervals for 3 months, starting 1 week before inoculation.

Approximately 3 months after inoculation the animals were killed and attempts made to recover *Y. enterocolitica* IX from the viscera and lymph nodes.

Rabbit antisera to *Br. abortus* strain 99 (serum RB 1) and to *Y. enterocolitica* IX (serum RY 1) were prepared by injection of pairs of adult rabbits with *ca.* 10^{10} of the respective organism emulsified in Freund's incomplete adjuvant.

A single dose of antigen was given divided over several intramuscular and subcutaneous sites and the animals exsanguinated 30 days later. Sera from each pair of animals were pooled.

In addition to these experimentally produced sera, samples of bovine diagnostic sera received for testing in connexion with the Brucella (Accredited Herds) scheme were available. Sixty samples of human serum taken at routine examination of laboratory workers were also examined for the presence of antibody to *Brucella* and *Yersinia*.

Absorption of sera

Antibodies reacting with *Br. abortus* were absorbed by mixing 1 ml. of serum with 2 ml. of a washed suspension of *Br. abortus* strain 99, containing *ca.* 10^{12} organisms/ml, and incubating at 37° C. for 1 hr. The absorbed serum was recovered by centrifugation. Antibody reacting with *Y. enterocolitica* IX was absorbed by an identical procedure except that a washed suspension of this organism was used.

Serological tests

Serum agglutination tests. Serum agglutination (SA) tests using 0·5 % phenol-saline as diluent were done according to WHO Monograph 19 (1953). The titres were expressed as reciprocals.

Indirect (antiglobulin) agglutination tests. These were performed according to the Coombs, Mourant & Race (1945) procedure as modified by Brinley-Morgan (1967). Tests with human and bovine sera were performed with rabbit antisera to human and bovine IgG globulins respectively. Tests with rabbit sera were performed with sheep antiserum to crude rabbit globulin fractions.

Rose Bengal plate test. Spot tests with RBPT antigen were performed on 0·03 ml. unit volumes of serum according to Brinley-Morgan, MacKinnon, Lawson & Cullen (1969).

Quantitative tests with RBPT antigens were performed by making serial doubling dilutions of serum in 0·85 % saline and shaking 0.03 ml. volumes with equal volumes of antigen on a white tile. The highest dilution showing visible agglutination within 4 min. was taken as the titre of the serum.

Complement fixation tests. These were done using 0·1 ml. volumes of reagents in WHO pattern agglutination trays. Both soluble and cellular antigens were used, standardized to optimal titre. For the test three 50 % haemolytic doses of complement were used and the sheep erythrocytes were sensitized with four 100 % haemolytic doses of haemolysin. Fixation was carried out at 37° C. for 60 min. or, in some cases, at 4° C. for 18 hr. Tests were read to an end-point of 50 % haemolysis.

All sera for CF tests were inactivated by heating at 56° C. for 30 min.

Immunodiffusion tests. These were performed according to Ouchterlony (1953). The diffusion medium was 0·8% agarose (L'Industrie Biologique Française, S.A., Gennevilliers) in 0·85 % saline containing 0·1 % sodium azide.

Fluorescent antibody staining. The indirect method was used on smears of heat-fixed organisms (Cherry, Goldman & Carski, 1960). Primary staining was done with specific bovine antisera and secondary staining with fluorescein isothiocyanate-labelled rabbit antibovine globulin serum (Difco Laboratories, Detroit). Preparations were examined with a Leitz Ortholux fluorescent microscope.

RESULTS

Examination of a large number of bovine sera taken at random from samples submitted under the Brucellosis (Accredited Herds) scheme showed that all sera containing agglutinins for *Br. abortus* also agglutinated standard *Y. enterocolitica*

IX suspensions to the same or higher titre. In addition a few sera giving negative reactions with brucella antigen agglutinated *Y. enterocolitica* IX suspensions (Table 1). These results parallel those reported by Ahvonen *et al.* (1969) for human sera from patients with confirmed or suspected *Y. enterocolitica* IX infection.

Table 1. *Serum agglutination test titres of* 151 *cattle sera to* Y. enterocolitica *IX and* Br. abortus 99

Serum agglutinating test titres	Number of sera at specified titre	
	Yersinia antigen	Brucella antigen
< 10	49	76
10	19	26
20	31	10
40	12	4
80	7	12
160	4	5
320	12	6
640	4	8
1280	7	3
2560	1	1
5120	5	0
	151	151

Titres are expressed as reciprocals.

Examination of sera from cattle inoculated with *Br. abortus* or *Y. enterocolitica* IX under laboratory conditions also confirmed the reciprocal cross-reactions between the two organisms. Irrespective of which of the two organisms the animals were inoculated with the titre of the agglutinins to *Y. enterocolitica* IX was always equal to or higher than that to *Br. abortus* (Table 2). Similar results were obtained irrespective of whether the agglutinating antigen suspensions were standardized in terms of packed cell volume or turbidity, suggesting that the difference was not simply the result of differences in cell concentration.

Antisera to *Y. enterocolitica* IX, also agglutinated standard suspensions of *Br. melitensis* 16 M, *Br. suis* 1330 and *Br. neotomae* 5K 33 to similar titres to *Br. abortus*. Antisera rendered monospecific for *Br. abortus* by absorption with *Br. melitensis* agglutinated *Y. enterocolitica* IX, but monospecific antisera to *Br. melitensis* did not.

Examination of antisera to *Br. abortus* and *Y. enterocolitica* IX by the antiglobulin test also indicated a reciprocal cross-reaction. As shown in Table 2 the net increase in titre relative to the SA test titre was essentially similar for both the brucella and yersinia inoculated groups when tested against either antigen.

The indirect fluorescent antibody (IFA) test, as expected, gave results compatible with the antiglobulin test. Both organisms showed bright peripheral staining with anti-*Br. abortus* and anti-*Y. enterocolitica* IX sera. There was no staining with pre-inoculation sera at the same dilution.

The results of CF tests with bovine antisera did not indicate complete reciprocal

cross-reaction between the two organisms. As shown in Table 3, sera from animals infected with either *Br. abortus* or *Y. enterocolitica* IX reacted with brucella antigens in CF tests. However, some cattle sera from both these groups failed to react with yersinia antigen in CF tests. In the case of high-titre rabbit antisera, positive reactions were obtained with yersinia antigens but titres were lower than those obtained with brucella antigens and titres to the soluble antigen were lower than those to the intact cell suspension. The use of the CF test did not allow the antibody responses to the two organisms to be distinguished.

Table 2. *Serum agglutination and antiglobulin test titres of anti-Brucella and anti-Yersinia sera to* Br. abortus *and* Y. enterocolitica *IX antigens*

Serum	Serum agglutination test		Antiglobulin test	
	Brucella antigen	Yersinia antigen	Brucella antigen	Yersinia antigen
S 19 calves			ND	ND
S 1	10	40	ND	ND
S 2	20	20	ND	ND
S 3	320	640	ND	ND
S 4	160	640	ND	ND
S 5	80	640	ND	ND
S 6	80	160	ND	ND
S 7	320	640	ND	ND
S 8	640	2,560	2,560	> 10,240
S 9	1,280	5,120	5,120	> 10,240
S 10	160	160	640	5,120
S 19 bullocks				
B 1	320	640	1,280	5,120
B 2	320	320	1,280	5,120
Field samples				
F 1	20	40	20	80
F 2	10	20	40	40
F 3	20	40	20	160
F 4	80	160	80	1,280
F 5	640	640	5,120	5,120
F 6	320	640	1,280	10,240
F 7	80	640	1,280	5,120
F 8	80	160	1,280	5,120
F 9	160	320	640	5,120
F 10	80	320	160	1,280
Yersinia bullocks				
Y 1	20	160	80	5,120
Y 2	40	640	320	> 10,240
Y 3	80	1,280	640	> 10,240
Y 4	80	640	320	> 10,240
Rabbit sera				
RB 1 (anti-Brucella)	640	1,280	2,560	5,120
RY 1 (anti-Yersinia)	2,560	10,240	> 10,240	> 10,240

ND = Not done.
Titres are expressed as reciprocals.

Sera from animals injected with either *Br. abortus* or *Y. enterocolitica* IX both gave positive reactions to the RBP test. Similar results were obtained using a Rose Bengal stained *Y. enterocolitica* IX antigen (RBY). However, it was thought that quantitative differences might exist in the reaction of *Brucella* and *Yersinia* infected animals to these tests.

Tests performed on serial doubling dilutions of sera from these groups of animals confirmed this (Table 4). All samples of sera from cattle and rabbits inoculated with *Br. abortus* gave titres to RBBr. and RBY which were either identical or showed a slightly higher titre for the RBBr. antigen. All samples of sera from cattle and rabbits inoculated with *Y. enterocolitica* IX gave titres to RBY antigen which were significantly higher than those to RBBr. Examination of

Table 3. *Complement-fixation titres of anti-Brucella and anti-Yersinia sera to* Br. abortus *and* Y. enterocolitica *IX antigens*

	Whole cell antigen		Soluble antigen	
Serum	Brucella	Yersinia	Brucella	Yersinia
S 19 calves				
S 1	2	—	—	—
S 2	2	—	—	—
S 3	32	16	16	4
S 4	16	8	4	—
S 5	64	32	16	4
S 6	64	32	64	8
S 19 bullocks				
B 1	128	64	32	8
B 2	128	64	16	8
Yersinia bullocks				
Y 1	2	—	—	—
Y 2	8	4	8	—
Y 3	16	8	8	4
Y 4	16	16	16	8
Rabbit sera				
RB 1	512	32	256	64
RY 1	512	256	512	256

Titres are expressed as reciprocals.

approximately 150 sera from cattle with positive titres for *Brucella* ($> 1/40$ in the SA test or $> 50\%$ fixation at 1/4 in the CF test) and which were in some cases confirmed by isolation of the organism, gave results similar to those obtained in this test with sera from animals experimentally infected with *Br. abortus*. No false positive reactions were given by either RBBr or RBY antigen in tests on approximately 100 brucella-negative cattle sera or on 60 brucella-negative human serum samples.

The specificity of the quantitative RB tests in distinguishing antibodies to *Brucella* and *Y. enterocolitica* IX was confirmed by cross-absorption tests performed on antisera to the two organisms. The results summarized in Table 4 showed that absorption of anti-yersinia sera with *Br. abortus* removed antibodies

reacting to this organism in the quantitative RBT but did not eliminate the reaction to RBY antigen although the titres were substantially reduced. Absorption of the sera with *Y. enterocolitica* IX eliminated antibody to both organisms.

Absorption of anti-brucella sera with *Y. enterocolitica* IX removed antibody to both organisms as did absorption with *Brucella*. Thus the cross-reactions were not completely reciprocal although compatible with the results obtained in quantitative RBP tests with the two antigens.

Table 4. *Quantitative Rose Bengal test titres of absorbed and unabsorbed antisera to* Br. abortus *and* Y. enterocolitica *IX*

	Unabsorbed		Brucella absorbed		Yersinia absorbed	
Serum	RB Br. titre	RBY titre	RB Br. titre	RBY titre	RB Br. titre	RBY titre
Cattle						
Anti-brucella sera						
B 1	32	16	—	—	—	—
B 2	32	32	—	—	—	—
F 4	4	4	—	—	—	—
F 5	64	32	—	—	—	—
F 6	32	32	—	—	—	—
F 8	4	4	—	—	—	—
S 5	4	4	—	—	—	—
S 6	4	4	—	—	—	—
S 9	64	64	—	—	—	—
S 10	8	4	—	—	—	—
Anti-yersinia sera						
Y 1	2	4	—	1	—	—
Y 2	2	32	—	4	—	—
Y 3	8	256	—	16	—	—
Y 4	8	512	—	16	—	—
Rabbit						
RB 1 anti-brucella	32	32	—	—	—	—
RY 1 anti-yersinia	32	512	—	32	—	—

Titres are expressed as reciprocals.

Brucella-absorbed anti-yersinia sera still agglutinated OH-suspensions of *Y. enterocolitica* IX indicating that the residual agglutinins not absorbed by *Br. abortus* were H-specific (Table 5).

Attempts were also made to distinguish the antibody response to the two organisms by immunodiffusion tests. These results shown in Pl. 1, figs. 1 and 2 confirmed the cross-reaction between *Brucella* spp. and *Y. enterocolitica* IX. In fig. 1 the reaction of soluble extracts of *Br. abortus* (*Br. ab.*) and *Y. enterocolitica* IX (*Y.e.*IX) is studied. The line pattern components (l.p.c.) 1 and 2 appear specific to the *Y.e.*IX–anti-*Y.e.*IX system, whereas l.p.c. 3 is common to the *Y.e.*IX–anti-*Y.e.*IX and *Y.e.*IX–anti-*Br. ab.* systems. L.p.c. 4, 5 and 6 appear specific to the *Br. ab.*–anti-*Br. ab.* system but l.p.c. 7 is common to both this and the *Br. ab.*–

anti-*Y.e.*IX systems. L.p.c. 3 and 7 appear to merge but do not give reactions of complete identity.

In fig. 2, the reaction of soluble extracts of *Y.e.*IX, *Br. ab.*, *Br. melitensis* (*Br. mel.*) and *Br. suis* with anti-*Y.e.*IX serum is studied. Multiple l.p.c. 1, 2, 3, 4, are seen in the *Y.e.*IX–anti-*Y.e.*IX systems; single l.p.c. 5, 6 and 7 are seen in the *Br. ab.*–anti-*Y.e.*IX, *Br. mel.*–anti-*Y.e.*IX and *Br. suis*–anti-*Y.e.*IX systems respectively. The l.p.c. in *Br. ab.* and *Br. suis* appear to correspond to components of low diffusibility, that in *Br. mel.* to a component similar to the cross-reacting antigen of *Y.e.*IX.

Table 5. *Serum agglutination test titres of absorbed and unabsorbed anti-yersinia and anti-brucella sera to O and OH* Y. enterocolitica *IX antigens*

	Unabsorbed titre		Brucella absorbed		Yersinia absorbed	
Serum	OH	O	OH	O	OH	O
Cattle						
Anti-brucella						
S8	2,560	2,560	—	—	—	—
F5	640	640	—	—	—	—
B1	640	640	—	—	—	—
S9	5,120	5,120	—	—	—	—
Anti-yersinia						
Y1	160	20	40	—	—	—
Y2	640	40	160	—	—	—
Y3	1,280	80	320	—	—	—
Y4	640	80	160	—	—	—
Rabbit						
RB1 anti-brucella	1,280	1,280	20	10	—	—
RY 1 anti-yersinia	10,240	2,560	1,280	10	20	—

Titres are expressed as reciprocals.
Negative results = < 10.

DISCUSSION

The results of the SA tests showed that the observations of Ahvonen *et al.* (1969) on human sera were applicable to bovine sera. They also confirmed that complete cross-agglutination occurs between *Br. abortus* and *Y. enterocolitica* IX. The tests performed with various *Brucella* species showed that the cross-reaction with *Y. enterocolitica* IX is common to all smooth strains of the genus *Brucella*. Thus the serological response to *Y. enterocolitica* IX cannot be distinguished from that to brucellas on the basis of SA tests. Similarly the results of the antiglobulin tests indicated that both organisms evoked cross-reacting 'incomplete' antibody thus rendering them indistinguishable on the basis of Coombs or IFA tests. The results of the CF tests were also inconclusive in this respect.

These results clearly indicated that the standard serological tests employed for the diagnosis of *Brucella* infections failed to differentiate the serological response to these from that to *Y. enterocolitica* IX. For this reason an attempt was made to

devise a test which would differentiate between the serological responses to the two organisms.

The results of immunodiffusion tests confirmed the cross-reaction between *Y. enterocolitica* IX and brucella strains but also showed that l.p.c. specific to each group could be detected with homologous antisera. Unfortunately this test was insufficiently sensitive to be of general use in evaluating field sera.

Spot tests performed with RBBr. and RBY antigens also confirmed the cross-reaction between the two groups. However, by performing the test on serial dilutions of serum it was possible to detect differences in titre between the *Brucella* and *Yersinia* groups. From the results obtained, it appeared that brucella-infected individuals gave titres to RBY and RBBr. antigen which were either equal or marginally higher for the brucella antigen. In no confirmed case of brucella infection was the reverse result obtained. On the other hand, *Y. enterocolitica* IX inoculated individuals gave titres which were invariably higher with the RBY antigen. Although the number of yersinia-inoculated animals studied was small, the results obtained were consistent and suggested that this test might be of value in differentiating the serological response to *Y. enterocolitica* IX from that due to *Brucella* spp. in cases of doubtful aetiology.

Hitherto there have been no reports of isolation of *Y. enterocolitica* IX from cattle in Great Britain and there is no evidence to suggest that such cross-reactions are likely to be encountered in field samples. With the exception of the examples cited by Mollaret (1968) and Goyon (1969) there seems little evidence to implicate *Y. enterocolitica* IX as a common cause of infection in cattle. In the present study, injection of *Y. enterocolitica* IX into cattle failed to produce any significant pathological changes apart from transient pyrexia and no organisms could be recovered post-mortem. Mollaret & Guillon (1965) also failed to produce any significant changes on inoculating a large number of strains of *Y. enterocolitica* into a wide range of animals.

However, in specific cases of doubt, the use of the quantitative Rose Bengal plate tests with RBY and RBBr. antigens, in combination with H-agglutination tests performed on brucella-absorbed sera, would enable a differential diagnosis to be made.

The authors would like to thank Dr W. J. Brinley-Morgan for helpful discussions. They would also like to thank Miss L. Brewer and Mr A. Feest for technical assistance, Mr R. Sayer of the Photographic Section, Department of Pathology, Central Veterinary Laboratory, for preparing Pl. 1, and Miss J. E. Shelton for characterizing the *Yersinia* strains used.

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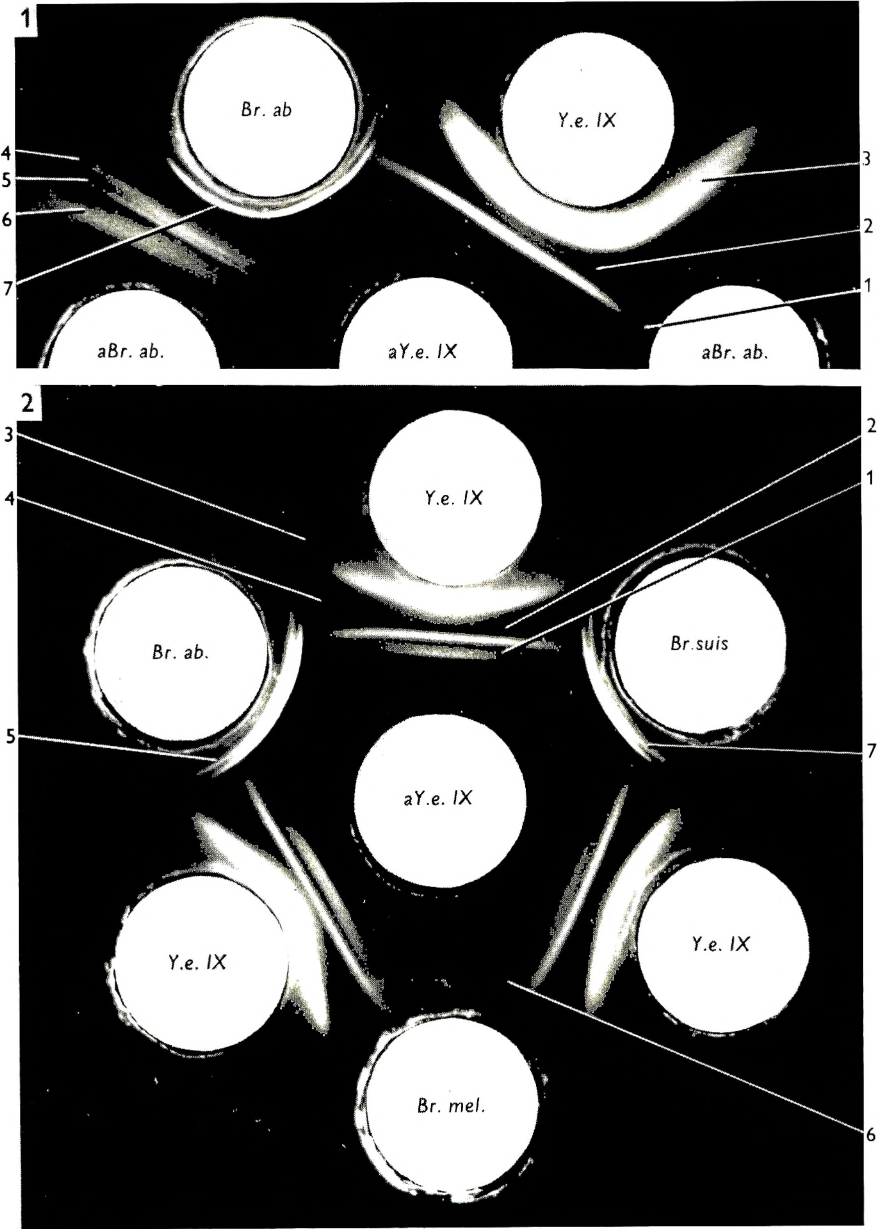
EXPLANATION OF PLATE

Plate 1

Fig. 1. This shows the reaction of *Br. abortus* (*Br. ab.*) and *Y. enterocolitica* IX (*Y.e.* IX) antigens with anti-*Br. abortus* serum (anti-*Br. ab.*) and anti-*Y. enterocolitica* IX serum (anti-*Y.e.* IX). L.p.c. 1 and 2 appear specific to *Y.e.* IX and l.p.c. 4, 5 and 6 appear specific to *Br. ab.* L.p.c. 3 is common to both the *Y.e.* IX–anti-*Y.e.* IX and *Y.e.* IX–anti-*Br. ab.* systems. L.p.c. 7 is similarly common to the *Br. ab.*–anti-*Br. ab.* and *Br. ab.*–anti-*Y.e.* IX systems. Thus l.p.c. 3 and 7 apparently correspond to serologically related but not identical components.

Fig. 2. This shows the reactions of *Br. ab.*, *Br. melitensis* (*Br. mel.*) and *Br. suis* antigens with anti-*Y.e.* IX serum. L.p.c. 1, 2 and possibly 4 are identified as specific to *Y.e.* IX. L.p.c. 5, 6 and 7 are given by reaction of *Br. ab.*, *Br. mel.* and *Br. suis* with a *Y.e.* IX serum and appear related to l.p.c. 3 of the *Y.e.* IX–anti-*Y.e.* IX system.


1
Br. ab
Y.e. IX
4
5
6
7
3
2
1
aBr. ab.
aY.e. IX
aBr. ab.
2
3
4
Y.e. IX
2
1
Br. ab.
Br.suis
5
7
aY.e. IX
Y.e. IX
Y.e. IX
Br. mel.
6

J. Hyg., Camb. (1970), **68**, 531
Printed in Great Britain

Staphylococcal infection in subdivided general surgical wards

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(*Received* 18 *May* 1970)

SUMMARY

The spread of *Staphylococcus aureus* was studied in three general surgical wards of identical design which consisted of a number of separate rooms, and the results were compared with those in the same unit previously studied, in subdivided wards elsewhere and in large open wards.

The nasal carrier rate of *Staph. aureus* by patients rose during their stay in the ward, but its peak rate was comparable with minimum rates reported in other subdivided wards and was lower than in open wards.

Staphylococcal sepsis rates were lower than in most open wards and were also much lower than those found previously in the same unit when overcrowding was common and each sex had its own ward.

INTRODUCTION

One of the features of recent hospital designs has been the adoption of separate rooms within each ward, mostly containing four patients or less. One reason for this is to reduce the level of cross-infection. This paper investigates the extent to which this has been successful in three general surgical wards in a recently built District General Hospital in Fife.

Architecture and ventilation

All three wards have the lay-out shown in Fig. 1. Wards A and B are on the first and second floors of the south wing and ward C on the second floor of the east wing of the building, which has three floors and a basement. There are one 8-bed, four 4-bed and four 1-bed rooms in each ward. The 8-bed rooms measure 45 ft. by 20 ft., the 4-bed 20 ft. square, and 1-bed 20 ft by 10 ft. Ceiling height is 10 ft. Each room has a door, which is usually left open. Ventilation is natural. Ward doors leading to the central staircase are normally shut, but those leading to the peripheral staircase are usually left open. Also included in the block are a paediatric ward on the ground floor, south wing, the pharmacy on ground floor, east wing and a urology ward and dermatology out-patient unit on the first floor of the east wing.

The west wing, not shown in Fig. 1, comprises the X-Ray department and offices on the ground floor, residency on the first floor and a suite of one small and two main operating theatres on the second floor, which serves the whole block. The theatres are ventilated by positive pressure at between 15 and 20 air changes per hour.

Floors were cleaned daily with a vacuum cleaner, and by mopping and polishing. Bed curtains were hot-laundered about once every 5 weeks and the cotton blankets after discharge of each patient. Otherwise, no special measures against cross-infection were in operation and the hospital did not have an active Control of Infection Committee during the investigation. There was no Control of Infection Officer or Sister.

Air movements were not studied in detail, but the presence of open doorways leading to the peripheral staircase must have led to a considerable exchange of air

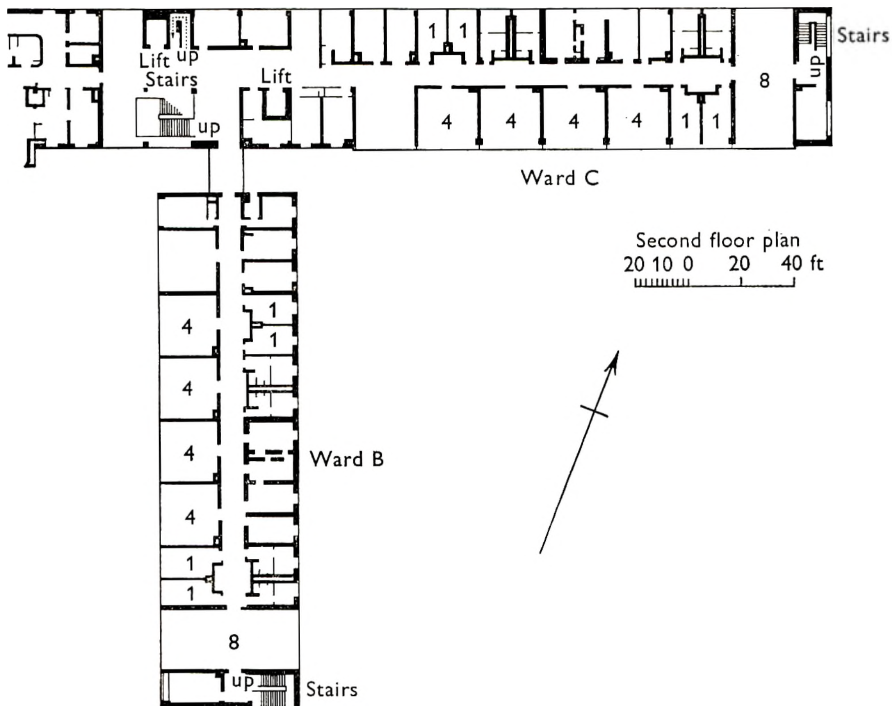

Fig. 1. Plan of wards B and C. Bed-rooms and their bed complements are indicated by the numerals 1, 4 or 8.

between wards A and B. The doors between the paediatric ward and the south and central stair wells were normally kept shut so that air from this ward was unlikely to move in large amounts to wards A and B above it. At the distal end of ward C the staircase led down to an outside door in frequent use by out-patients, so that considerable ventilation by fresh outside air must have occurred through the open doors at this end of the ward.

METHODS

Patients

In all three wards both 1-bed and 4-bed rooms were used for either males or females, but females always occupied the 8-bed rooms. In ward C female patients were sometimes admitted to the 8-bed room prior to undergoing an operation,

followed by transfer to one of the other rooms. In wards A and B most patients admitted to 8-bed rooms remained there throughout their stay. In all three wards there was little movement of patients from 1-bed or 4-bed rooms to other room types.

Wound dressings

In ward A, clean wounds were sprayed with Nobecutane but otherwise left uncovered. In the other two wards dressings were used, which were removed on the 2nd or 3rd day after operation. Stitches were removed on the 8th and 9th days.

Wounds were considered to be septic only when visible pus was present and this was recorded only in major clean or potentially infected operation wounds, e.g. hernia (clean) or uncomplicated appendicectomy (potentially infected). Wounds where gross fouling occurred from a contaminated viscus (e.g. ruptured appendix) or where pus was already present at operation, were excluded from the investigation. 'Staphylococcal other sepsis' was recorded in any clinical infection arising in hospital, other than wound sepsis as defined above, from which *Staph. aureus* was isolated in significant numbers. It thus included respiratory, urinary or cutaneous infections and infections of minor surgical wounds such as drip incisions. It did not include infections already present on admission. Ascertainment of sepsis was made by following up routine bacteriology specimens sent to the laboratory.

Antibiotic treatment

Patients in ward C having operations usually had penicillin and streptomycin both as a local wound application and intramuscularly for several days after operation. There was no routine prophylactic administration of antibiotics in the other two wards. The percentages of patients receiving antibiotics in wards A, B and C were 39·7, 23·2 and 34·1 respectively; and in all wards, 31·7.

Staff

Each ward had one consultant surgeon. In addition, there were 6 beds in ward A looked after by a consultant urologist. The medical staff included one registrar and one house officer in each ward and an additional senior house officer in ward A. There were a sister, two staff nurses, two state-enrolled nurses and eight nursing trainees and auxiliaries in each ward during the day, with two to three nurses at night. Other professional staff included three anaesthetists and one physiotherapist shared between the three wards. There were three domestic staff in each ward.

Bacteriological examinations

Nasal swabs were taken from patients on admission and thereafter weekly on a fixed day, and weekly from surgical, nursing and other staff of the ward. Swabs were cultured on phenolphthalein phosphate serum agar plates which were incubated in air at 37° C. Coagulase-positive staphylococci were phage-typed and tested for resistance to penicillin and tetracycline. Air-sampling was carried out weekly by exposing 13·5 cm. diameter phenolphthalein phosphate serum agar plates for 8 hr. in each room. Up to ten coagulase-positive cultures from each plate

were phage-typed and tested for resistance to penicillin and tetracycline. When more than ten colonies of *Staph. aureus* were present on a plate, strains were assumed to be distributed in the sample in the same ratio as in the ten colonies that had been phage-typed.

Method of analysis

Acquisition of *Staph. aureus* was measured by recording phage-types and antibiotic resistance of strains as they appeared in successive nasal swabs from the same patient. A strain appearing which had not been found in previous swabs or which was different in resistance or phage-type from that found previously was assumed to have been 'acquired'. The conventions adopted for times of acquisition between swabbings were those of Lidwell *et al.* (1966). The survey took place between November 1967 and April 1969.

RESULTS

Incidence of sepsis

Table 1 shows the incidence of post-operative staphylococcal wound sepsis in 11 different operation groups, arranged in increasing order of sepsis rates. There was no sepsis among 24 varicose vein operations; appendicectomy and hernia

Table 1. *Incidence of staphylococcal sepsis in operation wounds*

	Ward A		Ward B		Ward C		All wards	
Operations	No. of operations	% septic	No. of operations	% septic	No. of operations	% septic	No. of operations	% septic
Varicose veins	7	0	3	0	14	0	24	0
Appendicectomy	96	0	134	1·5	123	0	353	0·6
Hernia	24	0	81	1·2	52	0	157	0·6
Biliary tract	56	0	43	2·3	30	3·3	129	1·6
Prostatectomy	5	0	18	5·6	11	0	34	2·9
Thyroid	14	0	15	6·7	4	0	33	3·0
Gastro-duodeno-jejunal	87	2·3	65	6·2	80	1·3	232	3·0
Ilio-colic-rectal	70	2·9	47	6·4	37	0	154	3·3
Miscellaneous clean	90	3·3	92	6·5	84	0	266	3·4
Breast	45	2·2	31	9·7	39	0	115	3·5
Urological	17	11·8	3	0	5	0	25	8·0
Total operations	511	2·0	532	4·1	479	0·4	1522	2·2

operations each gave 0·6 % staphylococcal infections and the remaining groups gave rates increasing from 1·6 % for biliary to 8·0 % for urological operations. The rate for all operations was 2·2 %. Ward C had a markedly lower wound sepsis rate than wards A and B (0·4 % as against 2·0 and 4·1 % respectively). This could not be ascribed to the difference in numbers of the various operations performed on patients in the different wards.

Table 2 shows rates of wound and other staphylococcal infections per 100

admissions in the three wards. The greatest number of admissions was in ward A (1064) and the least in ward C (841). The incidence of post-operative staphyloccocal wound sepsis was 0·9 % in ward A, 2·3 % in ward B and 0·2 % in ward C. The corresponding percentages for other staphylococcal sepsis were 1·6, 1·7 and 2·7 and for wound and/or other staphylococcal sepsis 2·4, 3·9, and 2·7. Three per cent of patients in all three wards developed staphylococcal sepsis, 1·2 % having wound sepsis and 2·0 % other sepsis. Males and females were about equally represented in each of these sepsis classes (this is not shown in the table).

The incidence of staphylococcal other sepsis in patients who had been previously admitted within 6 months was 4·1 % while in those without previous admission it was only 1·7 %. This disproportion was most marked in the case of sepsis due to tetracycline-resistant (T) strains, the incidence being 2·8 % in patients with previous admission and 0·8 % in those without.

Table 2. *Staphylococcal infections arising in hospital*

		% Patients developing staphylococcal infection		
Ward	Admissions	Wound sepsis	Other sepsis	Wound or other sepsis or both
A	1064	0·9	1·6	2·4
B	958	2·3	1·7	3·9
C	841	0·2	2·7	2·7
All wards	2863	1·2	2·0	3·0

Nasal carriage of Staphylococcus aureus

The pattern of nasal carriage of *Staph. aureus* by patients in successive weeks of stay was somewhat different from that found by Lidwell *et al.* (1966) in a thoracic ward at St Bartholomew's Hospital of similar lay-out, or by Parker, John, Emond & Machacek (1965) in patients in 1- and 2-bed cubicles at Coppett's Wood Hospital. Figs. 2 and 3 show carriage rates which were considerably lower in patients on admission and in staff (both were 18·3 %) than in the other surveys. Thus, Lidwell *et al.* (1966) reported 38 % for patients and 39 % for staff; and Parker *et al.* (1965), 37 % for patients. In addition, the total carriage rate did not fall on successive weeks of stay as in those two surveys, but rose to a peak of 28 % in week 6, a level which was however lower than the 6-week minimum of Parker *et al.* and only a little higher than the minimum of about 25 % of Lidwell *et al.*

This difference lay chiefly in the very low incidence of penicillin-sensitive (S) strains on admission compared with that reported by the other workers. This fell still further during the patients' stay from 8·0 % on admission to 1·5 % in week 7. The incidence of tetracycline-resistant (T) strains was 1·7 % on admission, a somewhat lower level than in the other surveys, but rose steadily to 10·8 % at week 6 and a peak of 17·6 % at week 9. These rates were similar to the other surveys shown in Fig. 3 for wards divided into small rooms or cubicles (A–D) but were much lower than in the unmodified open wards shown in E–G.

The incidence of penicillin-resistant (P) strains rose *pari passu* with that for T strains during the first 6 weeks of stay. The rising graph of total carriage rates was therefore determined by a normally rising P and T strain graph modified by an exceptionally low S strain incidence throughout the patients' stay.

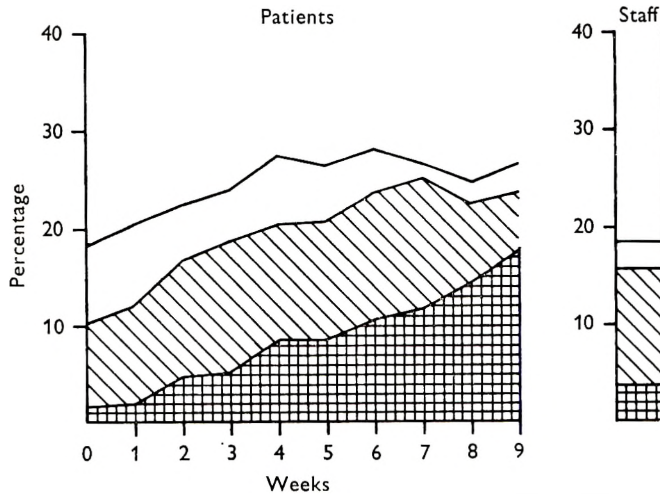

Fig. 2. Nasal carriage of *Staph. aureus* by patients during each week of stay in the three surgical wards. Average rate of nasal carriage by members of the staff. □, Sensitive to penicillin and tetracycline; ▧, resistant to penicillin only; ▦, resistant to tetracycline.

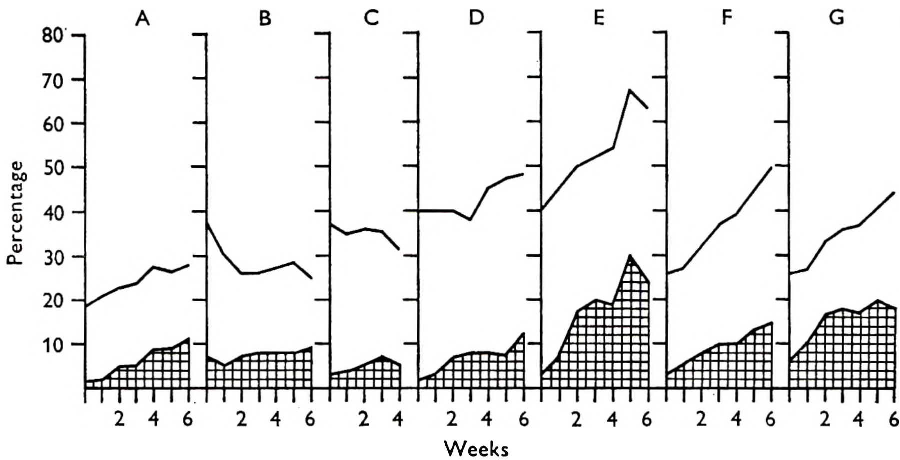

Fig. 3. Nasal carriage of *Staph. aureus* by patients in various hospital wards. Percentage positive in each week of stay in ward. Upper line: all strains of *Staph. aureus*. Lower line: tetracycline-resistant *Staph. aureus* [A, B, C, F and G: all tetracycline-resistant strains; D and E: all strains resistant to penicillin and tetracycline]. A, this investigation in all three wards; B, patients in similar lay-out, 1-bed, 4-bed and 10-bed rooms (Lidwell *et al.* 1966); C, patients in cubicles (Parker *et al.* 1965); D, E, two of three open surgical wards, D with four cubicles, E without (Williams *et al.* 1962). F, medical wards; patients not receiving antibiotics; G, medical wards; patients receiving antibiotics. (F, G, P.H.L.S. Cross-Infection Committee, results as given in Lidwell *et al.* 1966). Histograms B to G reproduced from Lidwell *et al.* (1966) by permission of Dr Lidwell.

The influence of previous admission to hospital on carriage rate was investigated in 440 patients. The incidence of S strains in these patients was 6·6 % and in patients not previously admitted, 8·4 %; that of P strains was 8·6 % in each group but there was a considerably greater incidence of T strains in previously admitted patients (3·4 %) than in those not previously admitted (1·3 %). This difference persisted for 2 weeks and then disappeared.

Weekly rates of carriage were calculated for the three wards A, B and C separately. Peaks occurred at different weeks in the various wards but the overall trend over 6 weeks was similar and average carriage rates were almost the same for all strains and for S and P strains. There was a somewhat higher average rate of T strains in ward A (5·0 %) than in ward B (3·4 %) or ward C (3·3 %) the excess occurring only in the first 3 weeks. For reasons of space the detailed figures for individual wards are not given here.

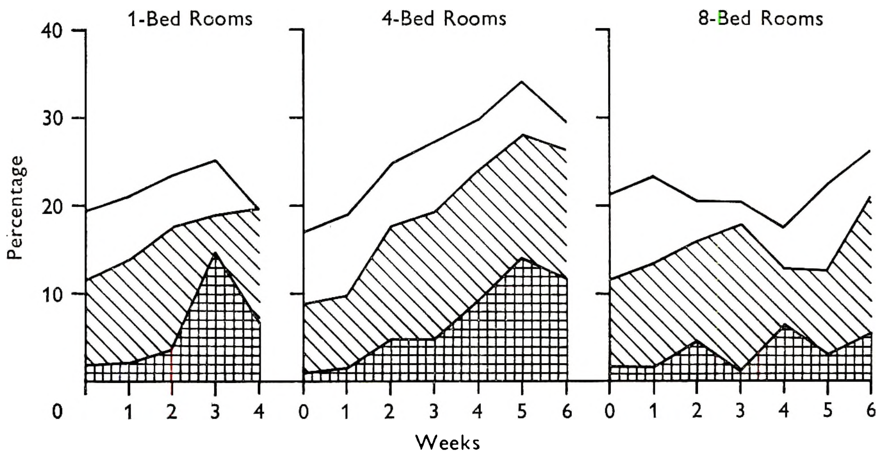

Fig. 4. Nasal carriage of *Staph. aureus* by patients in rooms with one bed, four beds and eight beds. Percentage positive in each week of stay in these rooms. □, ▧, ▦, as in Fig. 2.

Fig. 4 shows corresponding rates in relation to the type of room occupied by the patient throughout his or her stay. It includes only patients who had not been previously admitted to hospital within 6 months. The number of patients in 1-bed rooms became too small after 4 weeks to yield significant results but it is clear that a higher total carriage rate developed in 4-bed rooms than in either 1-bed or 8-bed rooms over the 4–6 weeks after admission. This was mainly due to a sharper rise in P + T strains in the 4-bed rooms than in the others. There was a higher average carriage rate of T strains in the 1-bed than in either 4- or 8-bed rooms during the first 4 weeks of stay, the figures for the three room types being 4·3, 3·0 and 2·7 % respectively.

Apparent acquisition of Staphylococcus aureus

Three hundred and seventy-one strains of *Staph. aureus* not found on admission were later isolated from nose swabs of patients. In 4574 patient-weeks exposure (see Table 3) the rate of acquisition was 8·6 per 100 patient-weeks, a figure rather

higher than the 6·4 found in patients in small cubicles by Parker *et al.* (1965), and more than double the figure of 3·4 found in patients in the subdivided thoracic ward of Lidwell *et al.* (1966). However, spurious acquisition of S and P strains due to failure to isolate an organism present on admission must be taken into account. Apparent acquisition of T strains is thought to be more significant than that of P and S strains and the present rate for T strains was 2·1 per 100 patient-weeks as against 1·4 for the cubicle patients and 1·3 for the thoracic subdivided ward patients. According to Parker *et al.* (1965) comparable figures for multiple-resistant organisms in large open wards range from 3·7 to 12 per 100 patient-weeks. Recently, however, the unusually low figure of 3·2 per 100 patient-weeks has been reported by Whyte, Howie & Eakin (1969) for T strains in open general surgical wards.

The effect of antibiotic treatment on acquisition rates is also seen in Table 3. The rate for all strains was highest (11·3 per 100 patient-weeks) in the patients receiving tetracycline only and the rate for T strains was also highest in this group at 4.0 per 100 patient-weeks. Rates for all strains were not much affected by other antibiotics, but T strain rates per 100 patient-weeks were 3·4 for those receiving antibiotics other than penicillin and tetracycline, 3·1 for patients receiving systemic antibiotics of all kinds, 2·7 for those having penicillin and 'other' antibiotics (i.e. not penicillin or tetracycline) 1·3 for penicillin only and 1·1 for those not receiving any antibiotics. Acquisition of S strains naturally tended to be less when antibiotics were given (1·3–2·9) than when no antibiotics were given (3·1). An exception was in the tetracycline only group when a rate of 4·0 per 100 patient-weeks was recorded.

Apparent acquisitions of *Staph. aureus* in relation to presumed sources are given in Table 4. In 233 strains no source was found within 10 days before the acquisition, and 42 were non-typable. Of the remainder, 72 strains were from known single-patient sources, of which 27 were T strains; 20 strains (including 6 T strains) came from a patient or patients in the same room as the recipient, 42 (15 T strains) from patients in other rooms, 27 (12 T strains) from staff and 14 (7 T strains) from patients in other wards.

In comparing rates of acquisition in relation to source with those reported by Lidwell *et al.* (1966), it seems best to pay attention mainly to rates for T strains (Table 4) as these are less likely to be spurious than those of P and S strains. The rates per 100 patient-weeks for patient-sources in the same room as the recipient (0·13), in other rooms (0·33), and from staff (0·26), compared with the corresponding figures derived from the above publication of 0·11, 0·29 and 0·14 respectively. The first two figures were similar in the two surveys, but the third figure, for staff sources, was larger in the present series than in the other.

Nasal acquisition of *Staph. aureus* from the 72 single-patient sources was further analysed according to the mobility and site of the source-patient and the room-type of the recipient in the week before the acquisition. Relatively few acquisitions (4) occurred from ambulant patient sources. The great majority were from patients who were up occasionally (35 total, 12 T strains) and from bed-fast patients (33 total, 13 T strains). On the other hand, 8 out or 12 source-patients who were in

Table 3. *Apparent acquisition of nasal* Staphylococcus aureus *by patients in relation to antibiotic treatment*

(Rate per 100 patient-weeks exposure. S = sensitive to penicillin and tetracycline, P = resistant only to penicillin. T = resistant to tetracycline.)

Antibiotic treatment and strains	No. of patient-weeks exposure	Rate of acquisition of strains
All patients	4574	—
All strains	—	8·6
S	—	2·5
P	—	3·9
T	—	2·1
No antibiotic	2156	—
All strains	—	8·1
S	—	3·1
P	—	3·9
T	—	1·1
Systemic antibiotic	2393	—
All strains	—	8·9
S	—	2·0
P	—	3·9
T	—	3·1
Penicillin only	77	—
All strains	—	6·5
S	—	1·3
P	—	3·9
T	—	1·3
Tetracycline only	150	—
All strains	—	11·3
S	—	4·0
P	—	3·3
T	—	4·0
Other only*	1365	—
All strains	—	9·0
S	—	1·9
P	—	3·7
T	—	3·4
Penicillin and other*	743	—
All strains	—	9·0
S	—	1·9
P	—	4·4
T	—	2·7
Tetracycline and other*	34	—
All strains	—	8·8
S	—	2·9
P	—	5·9
T	—	0

* Antibiotics other than penicillin or tetracycline.

other wards were either ambulant or up occasionally. Table 5 subdivides these figures. There was a predominance of T strain acquisitions in 1-bed rooms (6 among a total of 12 beds), as compared with 4-bed rooms (13 among 48 beds) or 8-bed rooms (8 among 24 beds). In both 4- and 8-bed rooms, the proportion of acquisitions of all strains from sources in other rooms to those in the same room was about 2:1; the corresponding ratios for T strains were 1·6:1 in 4-bed and 3:1 in 8-bed rooms. There was no greater tendency for patients who were up occasionally to act as other-room sources than patients who were bed-fast.

Table 4. *Apparent acquisition of* Staphylococcus ureus *in relation to* a *presumed source*

(Patient-weeks of exposure 4574.)

Source	No. of 'acquired' strains: Antibiotic resistance of strains S	P	T	All strains	Rate of acquisition per 100 patient-weeks: Antibiotic resistance of strains S	P	T	All strains
Known single patient	15	30	27	72	0·33	0·66	0·59	1·57
Patient(s) in same room	10	4	6	20	0·22	0·09	0·13	0·44
Patient(s) in other room	7	20	15	42	0·15	0·44	0·33	0·92
Patient(s) in same/other room	0	1	3	4	0	0·02	0·07	0·09
Patients or staff	1	3	1	5	0·02	0·07	0·02	0·11
Staff	4	11	12	27	0·09	0·24	0·26	0·59
No source within ten days	83	110	40	233	1·82	2·40	0·87	5·09
Non-typable	8	22	12	42	0·18	0·48	0·26	0·92
Patient(s) in other ward	1	6	7	14	0·02	0·13	0·15	0·31

Sources of staphyloccocal sepsis

Presumed sources of staphylococcal wound and other sepsis are shown in Table 6. In wound sepsis, the source was unknown in 13 out of 34 strains; of the remainder, the majority could be traced to other patients or staff (12), the rest coming from the patient himself (9). In other staphylococcal sepsis the majority of known-source strains came from the nose of the patient himself (16 out of 29). Eight strains originated from other patients, four from staff and one from patients or staff. About half of all 'other sepsis' strains had no known source (30 out of 59). There was a predominance of T strains in both types of sepsis.

Staphylococcus aureus *in the air*

Two thousand and thirty-one culture plates were exposed for 8 hr. each in the 27 rooms of the three wards. Two thousand and fifty colonies of *Staph. aureus* grew on these plates. Assuming that the number of bacteria-carrying particles settling on 1 ft.^{2} of culture medium per minute is approximately equal to the number per ft.^{3} of air (Williams, Blowers, Garrod & Shooter, 1966), this gives an approximate

Table 5. *Acquisition of* Staphylococcus aureus *from single-patient sources*

Mobility and site of source patient in week preceding acquisition		Room-type of recipient in week preceding acquisition of *Staph. aureus*: 1-bed, Antibiotic resistance of acquired strains: S	P	T	All strains	4-bed, Antibiotic resistance of acquired strains: S	P	T	All strains	8-bed, Antibiotic resistance of acquired strains: S	P	T	All strains
Ambulant	Same room as recipient	—	—	—	—	0	0	0	0	0	0	0	0
	Other room	0	0	1	1	(1)	1	(1)	3 (2)	0	0	0	0
Up occasionally	Same room	—	—	—	—	4	1	4	9	1	2	0	3
	Other room	1	2	4 (3)	7 (3)	1	9 (2)	3 (1)	13 (3)	0	2	1	3
Bed-fast	Same room	—	—	—	—	3	1	1	5	1	0	2	3
	Other room	1	2	1	4	1	6	4	11	1	4 (2)	5 (2)	10 (4)
Total	Same room	—	—	—	—	7	2	5	14	2	2	2	6
	Other room	2	4	6 (3)	12 (3)	3 (1)	16 (2)	8 (2)	27 (5)	1	6 (2)	6 (2)	13 (4)
	All rooms	2	4	6 (3)	12 (3)	10 (1)	18 (2)	13 (2)	41 (5)	3	8 (2)	8 (2)	19 (4)

Figures in parentheses are sources in other wards, and are included in 'other room' figures.

average air content of 49·5 *Staph. aureus*-carrying particles per 100 m.³ (14·0 per 1000 ft.³). This is less than half the average figure reported by Lidwell *et al.* (1966) of 124 per 100 m.³ (35 per 1000 ft.³), which was in turn much lower than the values observed earlier in open surgical wards at St Bartholomew's Hospital. Average counts were then between 635 and 776 per 100 m.³ air (180 and 220 per 1000 ft.³ (Noble, 1962).

Table 7 shows the average estimated air contents in the various wards and room types. Wards A and B were roughly equal (62·3 and 60·4 per 100 m.³ respectively), but ward C gave less than half of these (24·1 per 100 m.³). There were fewer *Staph. aureus* particles in the air of the 1-bed rooms than in 4- or 8-bed rooms. Total strains are given in Table 8 for each room type, the average numbers per 100 m.³ air for 1-, 4- and 8-bed rooms being 36·8, 60·2 and 53·8 respectively. Note the

Table 6. *Presumed sources of staphylococcal sepsis*

Type of infection		Presumed source: Self	Another patient or patients	Staff	Staff or patients	Not known	Total sepsis strains
Staphylococcal wound sepsis	S	0	0	1	2	4	7
	P	5	2	0	0	4	11
	T	4	6	1	0	5	16
	All strains	9	8	2	2	13	34
Staphylococcal other sepsis	S	1	1	0	0	3	5
	P	6	0	2	1	15	24
	T	9	7	2	0	12	30
	All strains	16	8	4	1	30	59

unexpected finding of the slightly greater number in 4-bed than in 8-bed rooms. There was a lower proportion of T strains to all strains in ward C than in wards A and B (Table 7) and this was true in each of the three room types.

The tendency of 1-bed rooms to yield fewer staphylococci than 4- or 8-bed rooms was reversed for T strains in ward B, there being 28·2, 19·9 and 12·2 colonies per 100 m.³ air in the three respective types of room. This was due to the exceptionally potent source-patient described below, whose room air yielded more than half the total T strains from 1-bed rooms in this ward.

Table 8 shows the distribution of sources of aerial strains. In each room-type the proportion of strains originating from single-patient sources in other rooms to those from the same room was about 1:2. This was the same for all rooms in ward A and B, but in ward C the ratio was 1:3. Other-room sources in this ward were therefore less important in aerial spread than in wards A and B.

The number of strains traced to single members of staff were between 2·7 and 3·8 per 100 m.³ air for the three types of room. The corresponding figures for wards A, B and C were 5·0, 4·1 and 0·7 respectively, the last figure being a particularly low one not only in relation to other wards, but also in relation to patient-sources in the same ward.

Table 7. *Airborne* Staphylococcus aureus *in relation to antibiotic resistance and ward and room-type where recovery occurred*

(Average estimated number of colonies per 100 m.^{3} air over the whole period of survey.)

Room type	Recovery from air in ward: A — Antibiotic resistance S	P	T	All strains	B — Antibiotic resistance S	P	T	All strains	C — Antibiotic resistance S	P	T	All strains
1-bed	6·5	18·2	14·0	38·7	2·6	23·8	28·2	54·6	4·5	7·3	3·1	14·9
4-bed	12·7	34·0	33·4	80·2	11·8	31·7	19·9	63·5	8·2	20·8	6·4	35·5
8-bed	11·6	29·1	32·2	72·8	11·8	42·0	12·2	66·0	6·2	10·8	2·5	19·5
All rooms	10·0	26·8	25·5	62·3	8·1	30·6	21·7	60·4	6·4	13·5	3·9	24·1

Table 8. *Airborne* Staphylococcus aureus *in relation to sources*

(Estimated colonies per 100 m^3 air. 1-b, 4-b, 8-b = 1-, 4-, 8-bed rooms.)

Location of source carriers.

Recovery from air	Single-patient sources						
Rooms	Same room	Other room	Other ward	Single staff sources	> 1 source	No known source	Total
1-b	11·5	5·4	0·8	3·7	4·2	11·1	36·8
4-b	15·0	6·3	1·4	2·7	3·4	31·4	60·2
8-b	12·6	7·2	0·5	3·8	2·2	27·6	53·8
Wards							
A	10·0	5·0	2·4	5·0	2·3	37·8	62·3
B	19·7	10·1	0·3	4·1	6·3	20·0	60·4
C	9·2	3·0	0·3	0·7	1·8	9·2	24·1
All rooms / All wards	13·1	6·1	1·0	3·3	3·5	22·6	49·5

Broadcasts of Staphylococcus aureus

The term 'broadcast' here means the isolation of ten or more colonies of *Staph. aureus* of a particular phage type and antibiotic resistance on any one weekly sampling day or 15 or more colonies on two successive sampling days. In ward A there were eight such broadcasts during the survey, yielding 152 colonies; in ward B there were twelve, yielding 454 colonies; and in ward C, four, yielding 80 colonies. Broadcasts were therefore largest and most frequent in ward B and smallest and least frequent in ward C. One of them came from an unusually potent source of staphylococci—a patient in a 1-bed room with tumour obstruction of the larynx and a tracheostomy wound infected with *Staph. aureus*. Over a period of 8 weeks no fewer than 146 colonies from this source were isolated from the routine weekly plates in ward B. There were two phage types involved, the first being a P strain, yielding 20 colonies, the second, a T strain, yielding 126 colonies. The latter strain was found in every room in the ward on one or more occasions and also in three rooms in ward A. Its highest concentration was in the patient's room, when on one occasion 70 colonies were estimated to be present on one 8-hr plate, corresponding to about 3300 staphylococcal particles per 100 m.^{3} air (970 per 1000 ft.^{3}). During the broadcast, the patient was either bed-fast or up occasionally.

Six of the broadcasts were of T strains, 14 of P strains and four of S strains. Apart from the patient described above, there was no obvious tendency for T strain broadcasts to be larger than those of P strains, but those of S strains were much smaller.

Two broadcasts of P strains were traced to staff, both nurses. One further broadcast came from either a patient or a nurse or both.

Spread of staphylococci between wards

One case of wound sepsis in ward B yielded a staphylococcus indistinguishable from a strain carried by a patient in ward A and by another in ward C, either of whom could have been the source. Nasal acquisitions from single-patient sources in other wards appeared to originate in ward A on two occasions, ward B on six occasions and ward C on four occasions. The direction of spread was from ward A to B once, A to C once, B to A twice, B to C four times and C to A three times.

Staph. aureus from sources in other wards was recovered from the air of bedrooms on a total of seventeen occasions, nine in ward A, four in ward B and four in ward C. Ward B was the source on seven occasions, spread occurring to ward A on six occasions and to ward C on one of these occasions. Ward C was the source on six occasions, spreading on three occasions each to wards A and B. Ward A was only once definitely shown to be a source, spread occurring to ward B.

DISCUSSION

The incidence of post-operative wound sepsis was lower than that found in the same unit in 1962, when 5·6 % of clean and potentially infected operation wounds and 8·3 % of all admissions to the general surgical wards developed staphylococcal sepsis (figures for unit (A) male and female wards, in Edmunds *et al.* 1965). This was a degree of sepsis commonly found in open wards, e.g. 5·2 % of operation wounds derived from the Report (1960) and 5·6 % of admissions from Williams *et al.* (1959). The present figures were 2·2 % for wounds and 3·0 % for admissions, clearly a considerable reduction, which can be attributed to the opening of an extension to the hospital shortly before the start of the survey; this enabled general surgical patients to be accommodated in three wards (A, B and C) with both sexes represented in each ward, instead of the two allocated to the speciality before (A, male, B, female). This prevented the overcrowding which was common before, and was associated with a nearly equal division of sepsis between the sexes, in place of the previous much higher incidence among the males.

The very low rate of staphylococcal wound sepsis in ward C (0·4 %) could have been related to the lower turnover of patients and greater stairwell ventilation by fresh air than in the other wards. However, the rate of staphylococcal other sepsis was higher than in other wards and nasal carriage rates were similar, so that one's attention was directed to possible differences in regimen affecting only those patients who had operations. The most likely seemed to be the routine administration of penicillin and streptomycin at and after operation in ward C. If this was a major cause for the low wound sepsis rate it would bear out the experience of Lidwell *et al.* (1966) that extensive use of antibiotics can under certain circumstances be a factor in reducing wound sepsis. The synergistic action of penicillin and streptomycin might well be effective even against penicillinase-producing staphylococci, but many people would not accept the risk of ototoxicity involved in the prophylactic use of streptomycin.

In common with other recent reports on subdivided wards, carriage rates were

considerably lower than those reported from large open wards. The influence of previous admission to hospital was not significant for S and P strains, but the carriage rate of T strains was 2·5 times higher in previously admitted patients than in the others; and this was associated with a higher staphylococcal sepsis rate, particularly due to T strains, in the former group.

An unexpected finding was that carriage rates of P and T strains rose more sharply in patients in 4-bed rooms than in 8-bed rooms. There was also a slightly higher average staphylococcal content of the air in the smaller rooms than in the larger. These facts may be related to the more severe illnesses and more intensive treatment of patients in the smaller rooms, and the exclusive occupancy of 8-bed rooms by females, who are less prolific sources of staphylococci than males (Blowers & McCluskey, 1965). In addition the air space per bed was greater than in other room types. These factors probably also explain the higher acquisition and average carriage rates of T strains in 1-bed rooms than in other rooms.

Nasal acquisition rates, both of antibiotic-sensitive and resistant strains were lower than in open wards, though not so low as those reported by Lidwell *et al.* (1966) in a similar architectural layout. The latter difference may be related to the smaller proportion of patients receiving antibiotics in this unit (37 %) as compared with the other (71 %).

Lidwell *et al.* (1966) found that there was more acquisition of staphylococci from patient-carriers in other rooms than from those in the same room as the recipient. This was confirmed in this investigation, the proportion of other-room to same-room sources of T strains being the same (about 2·5 to 1); however, nearly twice as many such acquisitions occurred from staff in this series as in the other.

It seems unlikely that the degree of ambulation of potential source carriers was of much importance in the spread of infection, as fully ambulant single-patient sources were in a very small minority, the great majority being up occasionally (i.e. for 2 hr. or less per day) or bed-fast. This leaves out of account the majority of acquisitions where patient sources were less well defined and it was therefore not possible to assess their degree of mobility. Nevertheless the ability of bed-fast patients to act as sources of staphylococcal acquisitions was noteworthy.

The low air counts of *Staph. aureus* particles may be explained in part by the long period over which the survey extended. The great majority of colonies were isolated on very few occasions while many weeks exposure yielded no colonies at all. Thus sampling limited to a shorter period might have yielded higher or lower average counts than that reported here, depending on whether or not broadcasts were included. In spite of this, however, the amount of aerial spread of *Staph. aureus* was clearly of a lower order than in open wards.

Ward C, in addition to a lower wound sepsis rate than wards A and B, had a lower average air count of staphylococci and was the site of fewer broadcasts. This was probably due to factors already suggested including a different antibiotic regimen, lower patient turnover and different ventilation, none of which was related to the architecture or design of the ward units themselves. On the other hand, average staphylococcal carriage rates were not significantly different in the three wards, and sepsis other than wound sepsis was more frequent in ward C than

in the other wards. In view of these facts it would seem desirable to study several identical units simultaneously if a proper assessment is to be made of the role of architecture in the spread of infection.

I am grateful to Mr J. W. M. Sutherland, Mr W. Drummond and Mr B. C. Abernethy for permission to study their patients, to the matron, sisters and nursing staff of the wards for their co-operation, to the Scottish Home and Health Department for financial support and to the data processing unit of the South Eastern Regional Hospital Board (Scotland) for help in analysis. I wish also to thank Professor R. E. O. Williams, Professor R. A. Shooter and Dr O. M. Lidwell for their advice and help, Dr J. D. Barrie for carrying on the project during my illness and Mr A. C. Heddle and Mrs M. Bailey for technical assistance.

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J. Hyg., Camb. (1970), **68**, 549
Printed in Great Britain

Determination of an optimal dilution of virulent feline infectious enteritis (panleucopaenia) virus for challenge purposes

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(*Received* 21 *May* 1970)

SUMMARY

When ten cats were infected orally with undiluted or a 10^{-1} dilution of virulent feline infectious enteritis (panleucopaenia) virus, all developed severe leucopaenia followed by the development of demonstrable antibody, but none died. Eighteen of 29 cats given a 10^{-2} dilution of virus died of the disease. Three of the survivors had white blood cell counts of less than 4000 and three had counts between 4000 and 6000 cells. Although the remaining five animals never had individual counts of less than 6000 cells, the geometric means of these counts showed that a marked depression in the leucocyte counts had occurred. All surviving cats developed antibody.

Among the ten cats dosed with either 10^{-3} or 10^{-4} dilution of virus, four died of feline infectious enteritis and three developed antibody after falls in the leucocyte counts. It is suspected that low dilutions of feline infectious enteritis virulent virus in cats produce a phenomenon similar to that reported by von Magnus (1954) with influenza virus in eggs.

Leucopaenia is commonly defined as less than 4000 white blood cells/mm.^{3} of blood. Counts lower than this are usual in cats which either die of the disease or have received large doses of virus; they are less common in cats surviving after administration of diluted virus. Challenge of cats with pre-existing antibody did not provoke a depression in the leucocyte counts.

INTRODUCTION

Over a number of years experimental infection of young cats with a 1/10 suspension of feline tissues infected with virulent feline infectious enteritis (FIE) (panleucopaenia) virus has produced leucopaenia in about half of the animals and death in less than one-quarter. These cats, aged between 10 and 16 weeks, were bred and reared in isolation, and were believed to be fully susceptible to the disease. Recently, O'Reilly, Paterson & Harriss (1969) have shown that, even in the absence of detectable antibody, not all cats will respond to a single dose of living attenuated FIE vaccine inoculated before the age of 12 weeks. Although these findings provide a partial explanation for the poor infection rate recorded above, they do not satisfactorily explain the low mortality rate. It was decided, therefore, to investigate the possibility that there is an optimal dilution at which virulent FIE virus should be used in order to cause the greatest number of deaths among antibody-free cats aged 12 or more weeks.

MATERIALS AND METHODS

Cats

The cats used in these experiments were bred in isolation at the Wellcome Veterinary Research Station, Frant, and all were 12 or more weeks old at the time of infection. Blood for leucocyte counts was obtained from the peripheral vein of the ear. When sera were required, the cats were bled from the jugular vein, using the method of restraint described by Hovell, O'Reilly, Calder & Povey (1970).

Experimental

Groups of five cats were used in the series of experiments to determine the dilution of virulent FIE virus that caused the highest mortality rate. Each cat was infected orally with 1·0 ml. of the appropriate dilution of virus, group A with 10^{0}, group B with 10^{-1}, group C with 10^{-2}, group D with 10^{-3} and group E with 10^{-4}. The groups were maintained in separate rooms and strict precautions taken to preserve isolation (O'Reilly, 1970).

Neutralization test

Sera were inactivated at 56° C. for 30 min., filtered and then stored at −20° C. until tested for neutralizing antibody by the method of O'Reilly *et al.* (1969).

Challenge virus

Small intestine, spleen and faeces from a naturally infected case of FIE were ground in a mortar with sterile sand and a 10 % suspension prepared in phosphate-buffered saline (PBS) containing 2000 units of penicillin and 1000 μg. of streptomycin/ml. The suspension was centrifuged at 3000 rev./min. for 15 min. and the supernatant stored at −20° C. Dilutions of virus were prepared in PBS.

RESULTS

The geometric means of the daily leucocyte counts of group A and group B cats showed a similar pattern of fall and rise after infection and have been combined (Fig. 1). During the first 4 days, the counts fell from 9000 to 4100. After a further 4 days, during which the counts did not exceed 5100 cells, there was a rapid increase. Although there were no deaths from FIE, three cats were destroyed on the 11th day because of severe respiratory disease and sera from these and the remaining animals (killed on the 15th day) had antibody to FIE virus (Table 1).

In group C a fall from 12,900 to 5300 in the geometric means of the leucocyte counts was seen between the 1st and 6th days after infection. The count then rose to 11,900 by the 10th day. On the next day, however, there was a drop to 7500 followed by a gradual rise during the next 2 days (Fig. 2). One cat died of FIE on the 7th day. When the four surviving cats were killed on the 15th day, three possessed significant amounts of antibody. The 4th cat's antibody titre (± 1/8) was of doubtful significance (Table 1).

In group D the geometric means of the leucocyte counts of the cats (Fig. 3) showed the same biphasic fluctuation as seen in group C. The initial fall by 5900

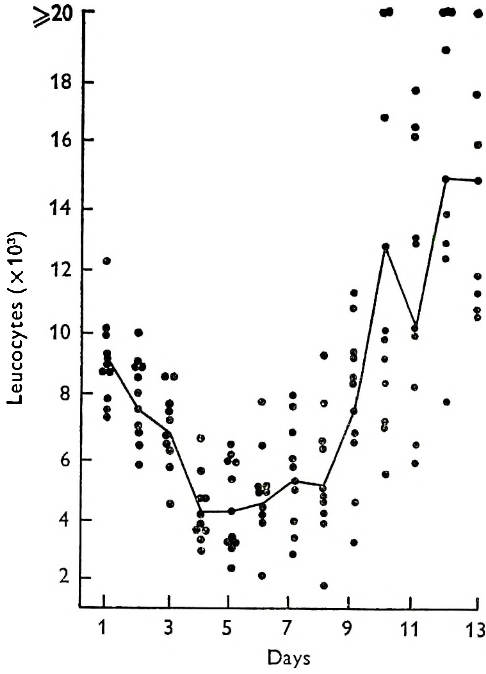

Fig. 1

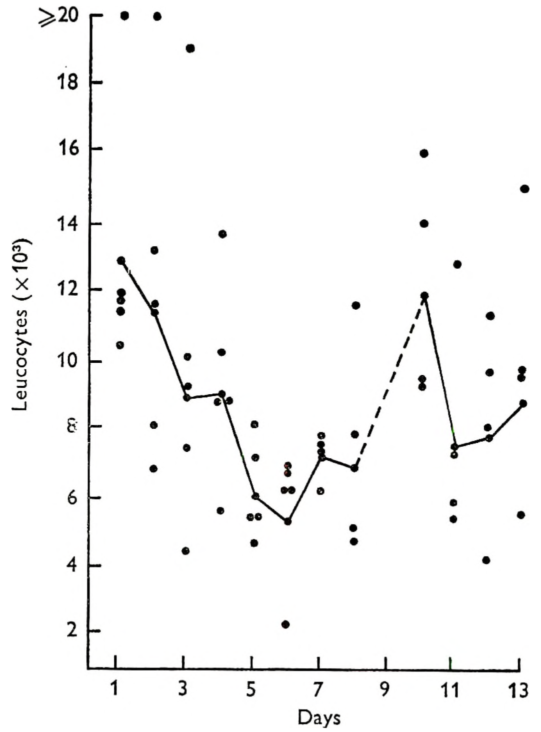

Fig. 2

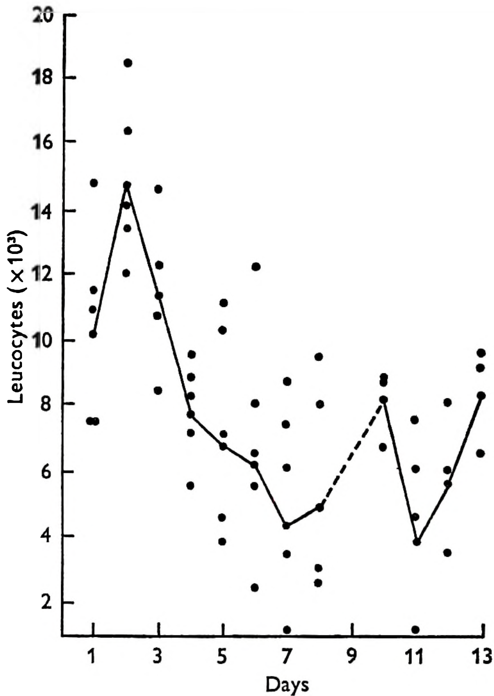

Fig. 3

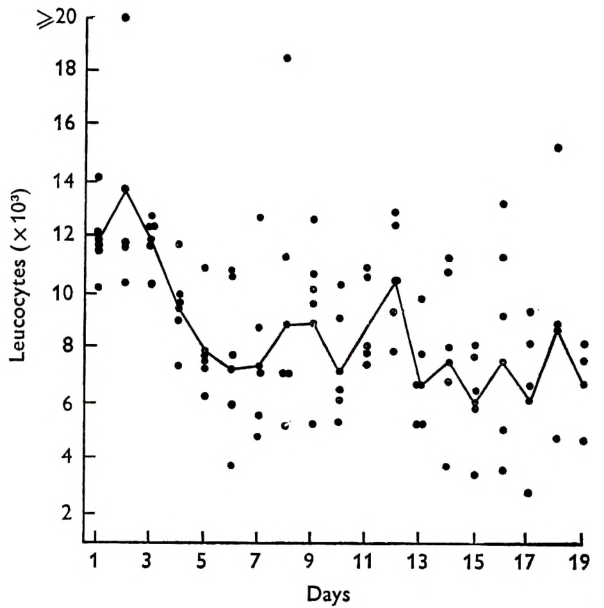

Fig. 4

Figs. 1–4. The individual and geometric means of daily leucocyte counts of cats infected orally with a suspension of tissues taken from a naturally infected case of feline infectious enteritis or a dilution of this suspension. Fig. 1. The pooled results of those given undiluted and 10^{-1} dilution (groups A, B). Fig. 2. 10^{-2} dilution (group C). Fig. 3. 10^{-3} dilution (group D). Fig. 4. 10^{-4} dilution (group E).

to 4300 cells during the first 7 days was followed by a rise to 8100 on the 10th day, after which another drop was observed. During the next 3 days, the count increased from 3800 to 8400. One cat died of FIE on the 8th day and another on the 12th day, and two of the three surviving cats had antibody when bled on the 15th day (Table 1).

Table 1. *Antibody responses of cats infected orally with different dilutions of virus*

Group	Dilution of virus suspension	Antibody titres after infection (days)				Remarks
		0	11	15	20	
A	10^0	< 8	—	128	—	—
		< 8	—	128	—	—
		< 8	—	128	—	—
		< 8	128	—	—	Killed—respiratory disease
		< 8	128	—	—	Killed—respiratory disease
B	10^{-1}	< 8	—	32	—	—
		< 8	—	32	—	—
		< 8	—	128	—	—
		< 8	—	128	—	—
		< 8	128	—	—	Killed—respiratory disease
C	10^{-2}	< 8	—	± 8	—	—
		< 8	—	32	—	—
		< 8	—	128	—	—
		< 8	—	512	—	—
		< 8	—	—	—	Died FIE day 7
D	10^{-3}	< 8	—	< 8	—	—
		< 8	—	128	—	—
		< 8	—	128	—	—
		< 8	—	—	—	Died FIE day 8
		< 8	—	—	—	Died FIE day 12
E	10^{-4}	< 8	—	—	< 8	—
		< 8	—	—	< 8	—
		< 8	—	—	8	—
		< 8	—	—	—	Died FIE day 17
		< 8	—	—	—	Died FIE day 18

In group E during the first 6 days after infection, the geometric means of the leucocyte counts fell from 12,000 to 7300 cells; thereafter, the count fluctuated between 6000 and 9000 (Fig. 4). Specific FIE deaths occurred on the 17th and 18th days and only one of the remaining three cats had antibody on the 20th day (Table 1).

Among the cats infected with undiluted or a 10^{-1} dilution of virus, there were no FIE deaths, but specific deaths did occur after the 10th day in cats infected with 10^{-3} or 10^{-4} dilution of virus. Since deaths were confined to the higher dilutions, the 10^{-2} dilution of virus was tested further to establish the suitability of this dilution for challenge purposes. The results now recorded were accumulated from a series of experiments and include the five cats in group C above. Twenty-nine susceptible cats were infected orally and 18 (62 %) died of FIE after showing clinical symptoms of the disease. Seventy-two per cent (13/18) of the deaths

occurred on the 7th and 8th days (Table 2). One cat died of pneumonia on the 14th day. When bled 11–18 days after infection, nine of the survivors had demonstrable antibody titres and the 10th cat (from group C) an indication of antibody (Table 3).

Table 2. *Distribution of specific deaths among* 29 *susceptible cats infected orally with* 10^{-2} *dilution of virulent FIE virus*

Days after infection	⩽ 5	6	7	8	9	10	⩾ 11
Number of cats dying	0	2	8	5	2	1	0

Table 3. *Number of days on which the leucocyte counts fell below* 6000 *cells/mm.*^{3} *in* 11 *cats surviving more than* 10 *days after infection with* 10^{-2} *dilution of virus, and the antibody titres*

Number of days on which leucocyte counts were			Post-challenge antibody*	
< 4000	4000–< 5000	5000–< 6000	Day of bleeding	Titre
4	1	1	11	128†
4	1	0	—	Died‡
3	0	0	18	128
0	1	2	15	± 8
0	1	1	15	128
0	1	1	15	512
0	0	0	15	32
0	0	0	12	32
0	0	0	15	128
0	0	0	12	128
0	0	0	12	8

* All cats were devoid of detectable antibody at challenge.
† Reciprocal of serum dilution.
‡ Died of pneumonia on the 14th day after infection.

Of the 11 cats which survived this challenge, three recorded leucocyte counts of less than 4000 and three between 4000 and < 5000. Fewer than 4000 leucocytes were seen most commonly in cats immediately before death and less frequently in those which survived. The five cats (Table 3) with counts in excess of 6000 all showed a depression in the number of circulating cells between the 4th and 7th days after challenge with the lowest mean count of 8200 on the 6th day (Fig. 5). The lowest single count recorded was 6200 on the 4th day and over the next 4 days, two other cats had counts in the region of 7000.

Twelve cats with antibody resulting from vaccination were challenged with 10^{-2} dilution of virus. Two of the cats had each received one dose of a live attenuated vaccine 21 days before challenge, and the remainder 2 doses of inactivated vaccine 42 and 21 days before challenge. Challenge of these cats with virulent virus produced neither leucocyte depression (Fig. 6) nor an anamnestic serological response (Table 4).

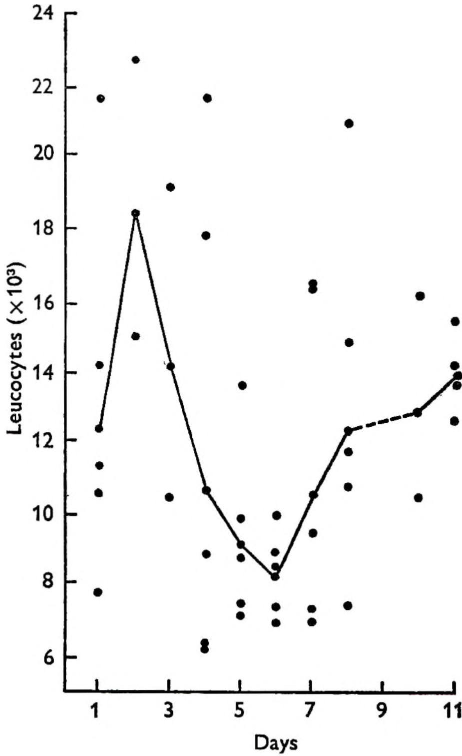

Fig. 5

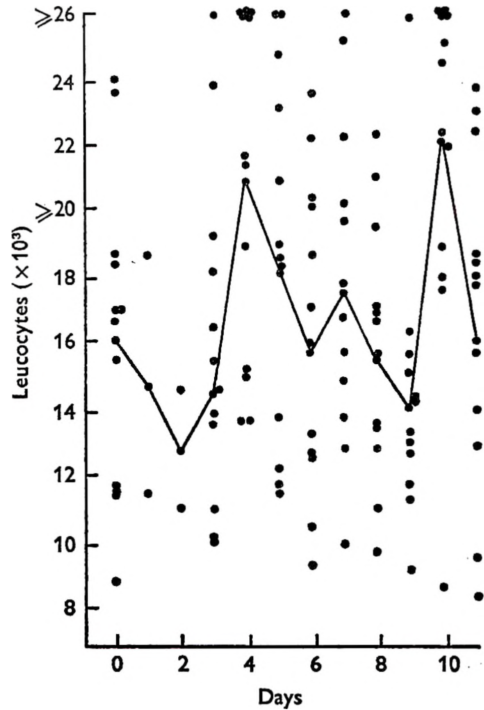

Fig. 6

Fig. 5. The individual and geometric means of leucocyte counts of five of eleven antibody-free cats whose leucocyte counts after challenge with 10^{-2} dilution of the suspension were depressed, but did not fall below 6000 cells/mm³.

Fig. 6. The individual and geometric means of leucocyte counts of 12 cats with pre-existing antibody before challenge with 10^{-2} of the suspension. Note that there was no depression of the leucocyte counts.

Table 4. *Antibody titres of* 12 *vaccinated cats before and after challenge with* 10^{-2} *dilution of FIE virus*

Type of vaccine	Time in days between pre- and post-challenge sera	Antibody titres	
		Pre-challenge	Post-challenge
1 dose of attenuated	14	512	128
		128	32
2 doses of inactivated	18	8	8
		8	32
		32	32
		32	128
		128	128
		128	128
		128	128
		128	128
		128	128

DISCUSSION

The oral administration of either undiluted or a 10^{-1} dilution of virus caused cats to become infected with FIE, as shown by the severe falls in the numbers of circulating white blood cells and the development of antibody. However, none of them died. On the other hand, the higher dilution of virus, which failed to produce antibody in all dosed cats, did cause some specific FIE deaths. These findings suggest that low dilutions of virus are less likely to cause mortality, possibly resulting from 'auto-interference'. Von Magnus (1954) first described a similar phenomenon when he injected undiluted influenza virus into the allantoic cavity of embryonating hens' eggs and found the harvested fluid had low infectivity but high haemagglutinating activity. Inoculation of high dilutions of influenza virus, however, yielded viral particles of high infectivity and haemagglutinin.

Many cats clinically ill with FIE will have leucocyte counts below 4000 cells; some will die and the others recover. Seventy-three per cent (8/11) of the survivors in the 10^{-2} dilution of virus challenge experiments never had leucocyte counts below 4000. Nevertheless, all survivors must have been infected, for they exhibited a depression in the white blood cell counts and subsequently developed antibody. Thus, it appears that infection of susceptible cats with FIE always causes a depression in the leucocytes, whereas challenge of cats with antibody does not provoke a drop in the total of the circulating white blood cells. Since a number of cats may show a rise in leucocytes on the 2nd day after infection (Figs. 3–5) (Riser, 1947), any subsequent depression should be related to the cell count on the 1st day after infection.

Among the ten cats dosed with 10^{-3} or 10^{-4} dilution of virus, there were three specific deaths which occurred after the 11th day. Lawrence & Syverton (1940) found virus present in experimentally infected cats on the 2nd, 3rd and 5th days but not on the 9th day after infection. O'Reilly (1970) has shown that virus was present in the tissues of a cat 4 days after exposure to infection and that an in-contact cage mate to another deliberately infected cat had a leucocyte count of 3800 five days after the infected cat was leucopaenic. He has also found antibody as early as 7 days after vaccination (unpublished results). Presumably, viraemia, as in most other virus infections, is of short duration and coincides with the fall in the leucocyte counts. It is reasonable, therefore, to assume that those cats which died 12, 17 and 18 days after dosing were secondary and not primary cases of FIE. Among the cats dosed with a 10^{-2} dilution of virus, that which had an antibody titre of ± 8 (Table 1) had its lowest leucocyte count on day 12 (Fig. 3). This cat may well have been a secondary case of FIE which would explain the low antibody titre when it was bled 3 days later.

In these experiments the optimal dilution of FIE virus giving an effective challenge was 10^{-2}. It caused depression of the leucocyte counts, specific deaths of more than 50% of the animals within the first 9 days and the development of antibody in the survivors.

The technical assistance of Mr W. F. Matchett, A.I.M.L.T., A.I.S.T. and Mrs L. M. Hitchcock is much appreciated.

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J. Hyg., Camb. (1970), 68, 557
Printed in Great Britain

Further investigations on the airborne excretion of foot-and-mouth disease virus

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(*Received* 22 *May* 1970)

SUMMARY

Calf thyroid tissue cultures were found to be the most sensitive system in detecting virus collected in a large volume air sampler from boxes, where cattle, sheep and pigs infected with A and C strains of foot-and-mouth disease (FMD) virus were housed. It was confirmed with all strains of FMD virus tested that pigs excreted the most virus followed by cattle and sheep, but there was variation between strains, the highest virus recoveries being obtained from animals infected with O_1 and C Noville viruses. The results are discussed in relation to outbreaks of foot-and-mouth disease in Great Britain since 1954.

INTRODUCTION

The airborne excretion of four strains of type O FMD virus from cattle, sheep and pigs has been reported by Sellers & Parker (1969). In the present paper these investigations have been extended to include measurement of virus excreted by animals infected with other types and subtypes of foot-and-mouth disease.

MATERIALS AND METHODS

Virus

Two strains of FMD virus type A (A_{22}, Iraq 24/26; A_5 Eystrup (Tübingen)) and two strains of type C (C Lebanon 3/69; C Noville) were used. They had been passaged in cattle apart from C Lebanon, which had been passaged once in IB-RS-2 cells (de Castro, 1964). The O_1 and O_2 strains of FMD virus shown in the Tables 1 and 5 had been passaged in cattle and pigs respectively.

Virus assay

The stock preparations of the virus strains were assayed by inoculating serial dilutions into unweaned mice (Skinner, 1951) and the following tissue culture systems maintained as monolayers in tubes: calf thyroid, BHK 21 Clone 13, IB-RS-2, primary guinea-pig kidney and calf kidney. In the first experiments air samples were assayed in these systems and also in monolayers in bottles up to the size of Roux bottles. In later experiments only calf thyroid tubes and unweaned mice were used.

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Infection of animals

The breeds and weights of the animals used have previously been described (Sellers & Parker, 1969). Sheep and pigs were inoculated subcutaneously on the coronary band and on the bulbs of the heel of one foot, respectively. Cattle were inoculated intradermally at four sites on the tongue. In one experiment (A_{22}) cattle were exposed to infection by being held in an isolation unit containing diseased pigs. In another experiment (A_{22}) cattle were inoculated intramuscularly. The dose of virus employed in all cases was from 10^5 to 10^6 TCD 50.

Air sampling was carried out as previously described (Sellers & Parker, 1969); the animals were examined daily and the extent of lesions recorded.

RESULTS

Comparative titres of stock preparations and of aerosol samples

The titres of the various virus stocks are shown in Table 1. It can be seen that calf thyroid tissue culture was the most sensitive system for virus detection. In a series of air samples tested in calf thyroid cultures and in mice, calf thyroid cultures gave a higher titre than mice on 33 out of 42 occasions. In addition minimal

Table 1. *Titres of different strains of FMD virus in various assay systems*

Assay system	Virus strains					
	O_1 BFS 1860	O_2 Brescia	A_5 Eystrup	A_{22} Iraq	C Lebanon	C Noville
Calf thyroid	7·7*	6·5	7·1	7·1	8·1	8·3
BHK 21	4·4	4·3	2·84	4·5	6·1	2·1
IB-RS-2	4·95	4·45	2·49	5·1	6·6	4·3
Guinea-pig kidney	5·9	5·53	5·15	6·6	7·5	7·05
Calf kidney	6·1	5·1	5·7	5·5	7·2	7·55
Unweaned mice	7·5	6·5	4·8	6·9	7·5	7·6

* Log ID 50 per ml.

amounts of virus were detected in 20 samples, which were negative in mice, the reverse occurring in only two samples. In some instances the failure to detect or the lower titres in calf thyroid cultures could be attributed to contamination after the first day. With A_5 virus calf kidneys were the most sensitive in some assays of aerosol samples and with C Lebanon (IB-RS-2 passage) on one occasion higher titres were found in BHK and IB-RS-2 cells than in calf thyroids. Otherwise calf thyroid cultures were more sensitive than BHK 21, IB-RS-2, guinea-pig kidney and calf kidney cultures, and only calf thyroid cultures together with mice were used in the later experiments.

Operation of large volume air sampler

To find the collecting fluid giving maximum virus recoveries the following fluids were compared during sampling of virus from infected pigs: phosphate buffered saline (PBS) containing 5% heat inactivated ox serum; PBS containing 0·5%

Table 2. *Extent of lesions and recovery of virus in the large volume sampler from infected cattle*

Strain and route	Days post infection	Extent of lesions: Tongue	Nasal discharge	Feet	Mouth	Primary	Generalized	Virus recovery: Range	Mean per animal
C Lebanon i/d tongue	1	2/2[b]	2/2	—	—	2/2	—	≤ 1·9*	≤ 1·6
	2	2/2[a]	2/2	2/8	—	—	2/2	2·9	2·6
	3	2/2	2/2	5/8	2/2	—	2/2	2·9	2·6
	4	2/2	2/2	5/8	2/2	—	2/2	3·0	2·7
C Noville i/d tongue	1	2/2[b]	—	—	1/2[a]	1/2	1/2	3·4	3·1
	2	2/2[a]	2/2	1/8	2/2	—	2/2	2·6	2·3
	3	2/2	2/2	4/8	2/2	—	2/2	3·6	3·3
	4	2/2	2/2	8/8	2/2	—	2/2	≤ 1·9	≤ 1·6
A_5 i/d tongue	1	2/2[b]	1/2	—	—	2/2	—	≤ 1·9–2·7	2·0
	2	2/2[a]	2/2	7/8	1/2	—	2/2	2·6–3·0	2·6
	3	2/2	2/2	7/8	2/2	—	2/2	2·4–3·1	2·45
	4	2/2	2/2	8/8	2/2	—	2/2	2·45	2·15
A_{22} intramuscular	3	1/2[a]	2/2	7/8	—	—	2/2	2·85	2·55
	4	2/2[a]	2/2	8/8	—	—	2/2	3·05	2·75
	5	2/2	2/2	8/8	1/2	—	2/2	2·95	2·65
	6	2/2	2/2	8/8	1/2	—	2/2	2·9	2·6
A_{22} exposed to infected pigs	3	—	1/2	1/8	1/2	—	1/2	≤ 1·9	≤ 1·6
	4	—	1/2	4/8	1/2	—	1/2	≤ 1·9	≤ 1·6
	5	—	1/2	4/8	1/2	—	1/2	≤ 1·9	≤ 1·6
	6	—	2/2	5/8	1/2	—	2/2	≤ 1·9	≤ 1·6
	7	2/2[a]	2/2	7/8	2/2[a]	—	2/2	3·1	2·8
	8	2/2[a]	2/2	8/8	2/2[a]	—	2/2	2·8	2·5

Numerator—no. of sites affected. Denominator—no. of sites available.
* Total virus (log ID 50) recovered over 60 min. at 1000 l./min.
[a] One of animals with unruptured vesicles.
[b] Both of animals with unruptured vesicles.

Table 3. *Extent of lesions and recovery of virus in the large volume sampler from infected sheep*

Strain	Days post infection	Extent of lesions: Inoculated feet, Heat	Inoculated feet, Lesions	Nasal discharge	Mouth	Other feet, Heat	Other feet, Lesions	Primary	Generalized	Virus recovery: Range	Mean per animal
A_5	1	7/8	1/8	2/8	—	—	—	7/8	—	≤ 1·9–2·1*	1·0
	2	1/8	7/8	2/8	1/8	1/24	—	6/8	1/8	≤ 1·9–2·6	1·2
	3	1/8	7/8	3/8	2/8	2/24	1/24	5/8	3/8	≤ 1·9–2·1	1·05
	4	1/8	7/8	2/8	2/8	3/24	1/24	5/8	3/8	≤ 1·9	≤ 1·0
A_{22}	1	7/8	—	1/8	—	—	—	7/8	—	≤ 1·9–2·25	1·1
	2	3/8	5/8	2/8	1/8	3/24	—	7/8	1/8	≤ 1·9–2·0	1·05
	3	1/8	7/8	2/8	1/8	5/24	1/24	6/8	2/8	≤ 1·9–1·95	1·0
	4	—	7/8	1/8	1/8	8/24	3/24	5/8	3/8	≤ 1·9	≤ 1·0
C Lebanon	1	5/8	1/8	2/8	—	—	—	6/8	—	1·6–2·4	1·0
	2	1/8	6/8	4/8	—	—	—	6/8	—	1·6–2·3	1·0
	3	1/8	6/8	4/8	1/8	1/24	—	6/8	1/8	≤ 1·9	≤ 1·0
	4	1/8	7/8	3/8	1/8	1/24	—	5/8	3/8	≤ 1·9	≤ 1·0
C Noville	1	8/8	—	1/8	—	—	—	8/8	—	4·6	3·7
	2	1/8	7/8	3/8	4/8	1/24	—	4/8	4/8	2·4	1·5
	3	—	8/8	6/8	5/8	1/24	—	3/8	5/8	2·2	1·3
	4	—	8/8	4/8	5/8	2/24	2/24	2/8	6/8	—	—

Numerator—no. of sites affected. Denominator—no. of sites available.

* Total virus (log ID 50) recovered over 60 min. at 1000 l./min.

Table 4. *Extent of lesions and recovery of virus in the large volume sampler from infected pigs*

Strain	Days post infection	Extent of lesions: Site of inoculation	Other feet	Mouth, tongue and snout	Primary	Generalized	Virus recovery: Range	Mean per animal
A_5	1	3/16	—	—	2/8	—	⩽ 1·9–2·7*	1·4
	2	10/16	8/24	2/8	2/8	6/8	2·4–5·6	3·1
	3	13/16	19/24	4/8	—	8/8	3·0–4·5	2·85
	4	14/16	20/24	6/8	—	8/8	1·4–4·4	2·0
A_{22}	1	3/16	—	—	2/8	—	2·25	1·35
	2	14/16	22/24	4/8	—	7/8	5·15	4·25
	3	14/16	22/24	4/8	—	7/8	4·4	3·5
	4	14/16	23/24	5/8	—	8/8	3·2	2·3
C Lebanon	1	4/16	—	—	3/8	—	3·3	2·4
	2	9/16	8/24	2/8	2/8	3/8	5·25	4·35
	3	12/14†	17/21	5/7	—	6/7	5·2	4·35
	4	12/14†	17/21	5/7	—	6/7	4·3	3·45
C Noville	1	3/15	—	—	3/8	—	—	—
	2	14/16	14/24	3/8	1/8	6/8	6·0–6·5	5·3
	3	16/16	18/24	7/8	1/8	7/8	5·8	4·9
	4	14/14†	21/21	7/7	—	7/7	—	—

Numerator—no. of sites affected. Denominator—no. of sites available.

* Total virus (log ID 50) recovered over 60 min. at 1000 l./min.

† One pig found dead.

bovine serum albumin; and M/15 phosphate buffer pH 7·2 containing 1 % heat inactivated ox serum. The highest recoveries, although only by a factor of 0·2–0·5 log units, were obtained using M/15 phosphate buffer and 1 % inactivated ox serum, and this collecting fluid was routinely employed.

Sampling of infected animals

The results of sampling the air in loose boxes containing cattle infected with different strains of FMD virus are shown in Table 2. Maximum recoveries were obtained when generalization of lesions was observed regardless of the initial method of exposure. In general the lowest amount of virus was recovered from animals infected with A_5 virus but apparently no detectable virus was recovered from one of the cattle exposed to A_{22} infected pigs. A feature of the clinical response was the occurrence of a serous nasal discharge at the time of primary lesions; later the discharge became more viscid and mucopurulent leaving encrustations on the nasal orifices. The development of other lesions is shown in Table 2.

With sheep (Table 3) the maximum amount of virus was found during the first 36 hr. after exposure, i.e. before lesions resulting from generalization were seen. Apart from the experiment in which C Noville was used, the mean recoveries per animal were lower than those obtained from cattle. Generalized lesions were not frequently observed; when they were, lesions on the dental pad were most common. An early serous nasal discharge was often observed.

Virus recoveries obtained in loose boxes containing pigs are shown in Table 4. As with cattle, maximum excretion occurred at the time of generalization. Little virus was excreted on the first day post inoculation, but on days two and three high levels of virus were detected, the virus titre decreasing on the fourth day and later. The extent of lesions is shown in Table 4. Nasal discharge was noted on occasions.

The experiments described here took place from May 1969 to March 1970 during spring, summer, autumn and winter and the temperature varied from 8·9 to 30° C. There was no apparent difference in the amount of virus recovered at the different times of the year. Relative humidity was consistently maintained above 70 %.

Maximum amount of virus recovered

The maximum amount of virus recovered for each strain and species, the extent of the lesions and the time after exposure are shown in Table 5. This also includes results obtained on other occasions with O_1 and O_2 strains of virus. In the last column the amounts are added together for comparison between strains.

DISCUSSION

Snowdon (1966) showed that the titres of unmodified cattle virus were on the average 30-fold higher in calf thyroid cultures than in other assay systems and in our experiments airborne FMD virus was recovered most consistently in calf thyroid cultures. Comparisons of the results obtained with different strains of virus is, therefore, based on the use of the most sensitive method of detection.

With all the strains the highest yield of virus was obtained from infected pigs, although there were variations between the amounts with the different strains,

the most coming from pigs infected with C Noville, O_1 and O_2. Less virus was detected in the collections from sheep and cattle, especially those inoculated with O_2, A_5, A_{22} and C Lebanon strains. It is not certain whether the variation between strains in the amounts recovered is due to better survival in air or to greater excretion from the animal, but the second reason is more probable owing to the close proximity to the animals of the collecting apparatus and the results obtained from detection of virus in the nose (Sellers, Donaldson & Herniman, 1970). When the log maximum titres are added together it is apparent that the greatest amount of virus was recovered from animals infected with the O_1 and C Noville strains.

Table 5. *Maximum amount of virus collected per animal, time and extent of lesions in animals infected with strains of FMD*

	Cattle		Sheep		Pigs		
Strain	Maximum virus	Time and extent of lesions (hr.)	Maximum virus	Time and extent of lesions (hr.)	Maximum virus	Time and extent of lesions (hr.)	Total virus collected
O_1	3·7*	41, G	3·6	24, N	5·4	45, G	12·7
O_2	2·5	42, G	2·1	22, N	5.1	68, G	9·7
A_5	2·8	46, G	1·7	44, P	4·7	48, G	9·2
A_{22}	2·8	70, G	1·35	19, N	4·25	48, G	8·4
C_{LEB}	2·7	93, G	1·5	19, N	4·35	46, G	8·55
C_{NOV}	3·3	70, G	3·7	22, N	5·6	46, G	12·6

* Total virus (log ID 50) recovered per animal over 60 min. at 1000 l./min.
N = no lesions. P = primary lesions. G = generalized lesions.

These strains are representative of the most recent epidemics in Europe since 1964, whereas of the other four strains, A_{22} and C Lebanon came from the Near East. It may be that this difference reflects a selection for ability to spread by the airborne route or by animal movement depending on the prevalent climatic conditions. As found previously, maximum virus was detected in pigs and cattle when lesions had generalized, whereas with sheep, apart from A_5, maximum virus was detected before vesicles appeared. A constant feature was the presence of a nasal discharge, which suggests infection of the upper respiratory tract.

In a previous reference to airborne transmission of virus, sheep were described as acting as maintenance hosts, pigs as amplifiers and cattle as indicators (Sellers & Parker, 1969). In the light of the results obtained with other strains and types of virus, primary and secondary outbreaks of FMD in Great Britain since 1954 have been examined to determine the role of the various species. It is apparent that the amount of virus released by a species of animal is only one of the factors influencing the degree of airborne spread after an initial outbreak. The numbers of animals involved, the period before the disease is reported as well as the topography of area, the livestock density and the climatic conditions are all important. Out of the 179 primary outbreaks from 1954 to 1967 only 81 spread (Report of Committee of Inquiry Part 2, 1968). Examples of spread attributed to pigs as a primary source were O in Shropshire 1967, Worcestershire 1967, Hampshire 1967, A in Caernarvon

1957 and Warwickshire 1961 and C in Essex 1956 and the West Riding of Yorkshire 1960. Attributions to cattle as a primary source were found in outbreaks in Somerset 1957 and Northumberland 1966 (O), Norfolk 1958 and Northumberland 1960 (A) and Buckinghamshire and Wiltshire 1956 (C). Primary outbreaks were rarely attributed to disease in sheep, apart from an O outbreak in Gloucestershire (1956) where pigs were also involved. However, in the 1967–68 O_1 epidemic 29 outbreaks occurred where sheep and cattle were affected at slaughter. In 15 of these the lesions in sheep were described as older, and these sheep could have transmitted the disease to cattle on the same farm. In other instances, on farms where only sheep were affected, outbreaks in cattle nearby could be attributed to spread from the sheep. Examination of spread of disease up to 14 days after primary outbreaks showed that where subsequent outbreaks were attributed to local spread, i.e. wind, movement of wild animals and birds etc., 83 % of outbreaks were in cattle, 14 % in sheep and 3 % in pigs. Assuming that cattle, sheep and pigs are equally at risk, these percentages are higher for cattle and lower for sheep and pigs than might be expected from the numbers of animals involved. However, this distribution would support the suggestion previously made (Sellers & Parker, 1969) that signs of foot-and-mouth disease might be seen first in cattle since cattle would sample more of the infected air than sheep and pigs and since in cattle the rate of development of lesions is more rapid.

Mr W. G. Parkinson of the Ministry of Agriculture, Tolworth, Surrey, is thanked for his extensive help in supplying information on foot-and-mouth disease outbreaks since 1954. Mr N. S. Needham of the Ministry of Agriculture, Worcester, kindly gave us information on the outbreak in Worcester 1967. We should like to thank Miss C. D. Mills, Miss J. Piper, Miss J. Slater, N. H. Cheale and D. R. Taylor for their technical assistance.

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Printed in Great Britain

Inhalation, persistence and dispersal of foot-and-mouth disease virus by man

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(*Received* 22 *May* 1970)

SUMMARY

Sampling of human subjects, who had been in contact with animals infected with foot-and-mouth disease (FMD) virus, showed that virus could be recovered from the nose, throat, saliva and from air expelled during coughing, sneezing, talking and breathing. The amounts of virus recovered paralleled those collected with a large-volume sampler and multistage impinger and these findings confirmed that the highest recovery of airborne virus was from infected pigs followed by cattle and sheep. More virus was found in the noses of those examining infected animals than in those operating the samplers, but there was variation between the subjects. In the majority there was a 1·8 log fall in titre by 3·5 hr., but virus persisted in the nose of one subject for 28 hr. Nose blowing or washing the nostrils did not remove virus completely, nor were cloth or industrial masks completely effective in preventing inhalation of virus. It was possible to transmit virus from infected subjects to others on one occasion. No clinical cases of FMD in man resulted from exposure, nor was there any rise in antibody. Use was made of these findings in determining sites of aerosol excretion in animals, and the results are discussed in relation to FMD in man and to the spread of respiratory viruses by the airborne route.

INTRODUCTION

In previous papers (Sellers & Parker, 1969; Donaldson, Herniman, Parker & Sellers, 1970) we gave the results of sampling air in boxes where cattle, sheep or pigs infected with foot-and-mouth disease were housed. During the sampling period one or more of us and our assistants had to remain in the box for up to 1 hr. to operate the large-volume sampler. At completion we sampled our noses, throats and saliva, and found FMD virus. These findings led to further investigations on the amount, persistence and dispersal of virus by man, and the results are recorded in this paper.

MATERIALS AND METHODS

The types of animals, methods of infection, air sampling and virus assay of aerosols from infected animals have already been described (Sellers & Parker, 1969; Donaldson *et al.* 1970).

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Viruses

The strains of FMD virus used were: O_1 BFS 1860, O_1 Swiss 1/66, O_2 Brescia, A_5 Eystrup (Tübingen), A_{22} Iraq 24/64, C Lebanon 3/69 and C Noville.

Collection of samples

Materials from the nose and throat were collected on dry bacterial swabs or cotton buds which took up 0·001 to 0·059 g. (mean 0·021 g.) of secretion and placed in 4 or 5 ml. of phosphate buffered saline (PBS) containing 0·5% serum bovine albumin (PBS solution). Saliva (volume 0·1–0·45 ml., mean 0·24 ml.) was collected in 1 oz. bottles and diluted to 5 ml. with PBS solution. Plastic bags, into which people had sneezed, coughed, talked or breathed, were washed with 10 ml. PBS solution. Paper handkerchiefs used for nose blows contained 0·005–0·435 g. (mean 0·137 g.) of secretion and were placed in 1 oz. bottles containing 10 ml. PBS solution. On occasions nasal washings were obtained by introducing 2 ml. of PBS solution into each nostril. Samples were kept at 4° or – 70° C. until assayed in calf thyroid cell cultures or unweaned mice.

Serum was collected at intervals from people exposed to infection and was tested for neutralizing antibody by the constant serum-varying virus method. Mixtures of virus and serum were held at 4° C. for 24 hr. before inoculation into unweaned mice.

RESULTS

Comparison of methods of sampling

Table 1 contains the results of several experiments, where the air in boxes holding infected animals was sampled with the large volume sampler, by nasal swabs and on one occasion with the multistage impinger. The titre of the virus recovered in the nasal swabs from people examining animals (examiners) was on all occasions higher than from people operating samplers (collectors) in the box, as might be expected from the close proximity to the animals together with the disturbance created during capture and restraint. The difference varied from 0·4

Table 1. *Comparison of virus recovery by various methods*

			Virus recovery			
					Nasal swabs	
Species	Strain	Hours after infection	Large vol. sampler	Multistage impinger	Collectors	Examiners
Pig (8 pigs)	O_1 Swiss	70	6·3*	Stage 1, 3·95* Stage 2, 3·8 Stage 3, 3·55	3·4*	4·1*
	A_5	46	5·6	—	3·3	3·7
Sheep (8 sheep)	O_2	22	3·0	—	1·2	1·6
	C Noville	22	4·6	—	1·4	3·9
Cattle (2 cattle)	A_5	46	3·0	—	0·9	1·5
	C Noville	70	3·6	—	1·0	2·3

* Log ID50 per collection.

to 2·5 log. units; over a number of experiments the average difference was from 5- to 11-fold. The amounts of virus recovered from collectors paralleled those found with the large volume sampler; namely that most virus was in the boxes containing infected pigs followed by those containing cattle and sheep. The 2·3–3·2 log. units difference between the two methods was greater than the two log. units expected from the collecting rates: i.e. the large volume sampler operating at 1000 l./min. as compared to man breathing at 10 l./min. However (in Table 2) it is shown that the amount of virus in the nose did not increase after the first 5 min. of exposure probably owing to equilibrium between intake and clearance. There was variation between the amounts recovered from individual collectors and examiners of the order of 30- to 40-fold (Table 3).

Table 2. *Titres of virus recovered at intervals by various methods from eight pigs infected with O_1 Swiss 1/66*

Time of collection (min.)	Virus recovery			
	Large vol. sampler	Multistage impinger	Nasal swab	
			Collectors	Examiners
5	—	—	3·6*	—
10	—	—	3·0	—
15	5·1*	—	3·3	—
30	5·7	—	—	—
45	5·5	Stage 1, 4·15* Stage 2, 3·0 Stage 3, 3·2	3·0	After 45 min. 4·2

* Log ID 50 per collection.

Table 3. *Variation in amount of virus recovered from the nose*

	Nasal swabs	
Collectors:	2·1*, 2·5, 2·9, 2·9, 3·1, 3·3, 3·3, 3·7	Mean 2·98
Examiners:	2·9, 3·15, 3·65, 4·15, 4·4	Mean 3·65

* Log ID 50 per sample.

Dispersal of virus by various activities

As well as the taking of nasal swabs after examination of pigs, noses were blown, throats swabbed and saliva collected: plastic bags were used to collect the air from sneezes, coughs, talking and breathing. Some of the results are shown in Table 4. The amount recovered from nose blows was similar to that recovered in nasal swabs, whereas less virus was found in the throat and saliva. Virus was recovered on many occasions from coughs, sneezes, talking and breathing (Table 5).

Persistence of virus in nose

When subjects remained in the vicinity of infective animals, the concentration of virus found in the nose did not vary greatly (Table 2). In further experiments nasal swabs were taken at intervals after exposure to infected animals. Between

the first and second nasal swab the subjects had had a shower and made two changes of clothing. The rate of disappearance of virus varied from person to person. On average there was a fall in virus titre of 1·8 log. units in 3·5 hr. (Table 6). With one person virus was recovered 28 hr. after exposure, but no virus was recovered at 48 hr.

Table 4. *Amounts of virus recovered in nasal swabs, nose blows, throat swabs and saliva*

	Nasal swabs	Nose blows
Expt. 1	2·65*, 3·4, 3·65, 4·15 Mean 3·46	2·7, 3·2, 3·25, 4·15 Mean 3·33
Expt. 2	3·5, 3·8, 4·7, 4·7 Mean 4·17	3·4, 4·2, 4·4, 4·6 Mean 4·15

	Nasal swabs	Throat swabs	Saliva
Expt. 3	4·1, 4·1, 4·1	⩽ 0·9, ⩽ 0·9, 1·1	1·1, 2·3, 1·7
Expt. 4	2·7, 3·1, 3·1, 3·3	1·3, 1·3, 1·75, 2·5	—

* Log ID 50 per sample.

Table 5. *Dispersal of virus by various activities*

		Mean
Nasal swabs	3·1*, 4·15, 4·2, 4·4	3·96
Coughing (3 times)	⩽ 1·2, 2·2, 1·8, 2·4	1·9
Nasal swabs	2·6, 3·65, 4·7	3·65
Sneezing (3 times)	⩽ 1·9, 3·2, 3·4	2·8
Nasal swabs	2·5, 2·7, 3·0, 3·1, 3·5	2·96
Talking (1 min.)	⩽ 1·2, ⩽ 1·2, 1·4, ⩽ 1·2, 3·6	⩽ 1·72
Nasal swabs	2·7, 2·7, 3·6, 3·7, 3·8	3·3
Breathing (1 min.)	⩽ 1·2, ⩽ 1·2, 2·6, ⩽ 1·2, 2·6	⩽ 1·76

* Log ID 50 per sample.

Table 6. *Clearance of virus from nose*

		Time after exposure (hr.)			
	Subject	0	2·5–4·5	22–24	28
Expt. 1	1	2·1*	⩽ 0·9	⩽ 0·9	⩽ 0·9
	2	2·5	⩽ 0·9	⩽ 0·9	⩽ 0·9
	3*a*	2·9	1·1	⩽ 0·9	⩽ 0·9
	4*a*	2·9	1·5	⩽ 0·9	⩽ 0·9
	5*a*	3·1	1·1	⩽ 0·9	⩽ 0·9
	5*b*	3·1	1·5	⩽ 0·9	⩽ 0·9
	6	3·3	1·1	⩽ 0·9	⩽ 0·9
	7	3·3	1·2	⩽ 0·9	⩽ 0·9
	4*b*	3·5	1·3	⩽ 0·9	⩽ 0·9
	8	3·7	1·0	1·2	1·3

		0	1	2	3	5
Expt. 2	5*c*	4·1*	3·0	—	2·3	1·75†
	3*b*	4·1	2·3	1·7	⩽ 0·9	1·1†

* Log ID 50 per nasal swab. † Log ID 50 per nasal washing.

Removal of virus from nose

Various methods of removal of virus from the nose were attempted. After one nasal swab, the titre fell by 0·35 to 0·75 log. units: after swabbing the nostrils with swabs soaked in 1/1000 citric acid or tap water a loss of 0·6–1·0 log. units was noted. A single nose blow resulted in a reduction of 0·5 log. units but even after 10 nose blows virus was still detectable (Table 7). Wearing a surgical cloth* or an industrial gauze and cotton wool† mask reduced the amount of virus inhaled by 0·9 or 0·8 log. units (Table 8) but paper* masks had no effect.

Table 7. *Effect of nose blowing on virus remaining in nose*

	Before nose blow	After 1 nose blow	After 10 nose blows
Expt. 1	3·0*, 3·15, 3·4, 3·65 Mean 3·3	2·65, 2·4, 2·9, 3·15 Mean 2·8	—
Expt. 2	2·5, 2·7, 2·9, 2·9, 3·2 Mean 2·8	—	1·3, 2·0, 1·8, 2·5, 1·9 Mean 1·9

* Log ID 50 per nasal swab.

Table 8. *Effect of masks in preventing inhalation of virus*

Type of mask			Mean
Cloth	With	2·6*, 3·1, 3·2	3·0
	Without	3·6, 4·2, 3·9	3·9
Industrial	With	1·8, 2·8, 2·8	2·5
	Without	2·8, 3·4, 3·6	3·3

* Log ID 50 per nasal swab.

Transfer of virus

Four attempts were made to transfer FMD virus. Three people examined infected pigs and then talked with colleagues in a box at the other end of the isolation unit for two minutes on two occasions. In three of the four attempts no virus was recovered before or after exposure, but on one occasion $10^{1 \cdot 3}$ ID 50 was recovered from the nose of a recipient after the period of four minutes.

Antibody titres in persons exposed to FMD virus

Serum was taken from subjects before and 3 weeks after exposure. No significant titre or rise of antibody was detected, although one person showed neutralizing titres of 2·4 and 2·6 respectively before and after exposure to C Noville virus. No activity against FMD virus was found in nasal washings. Exposure to FMD virus did not prevent the development of respiratory illnesses in five out of eight subjects.

* Robinson and Son Ltd., Chesterfield.
† Martindale Electric Co. Ltd., London, N.W. 10.

DISCUSSION

It is not surprising that after exposure to infected air FMD virus was found in the nose. The amounts found can be correlated with those recovered by other means provided that allowance is made for the rapid uptake by the nose and subsequent clearance. The results obtained from nasal swabs confirm those previously found with the large volume sampler, namely that the greatest amount of airborne virus is excreted by pigs, followed by cattle and sheep, and that sheep excrete virus although no lesions are apparent.

The higher amount of virus collected during examination of the animals may be attributed to closer proximity and the disturbance set up in catching and holding the animals. Use was made of the greater mobility of the nose compared with that of the large volume sampler in assessing the sites of origin of the airborne virus. In preliminary experiments we found that even when extensive vesicles were present on the tongue of steers the day after inoculation, no virus was recovered from the nose of an examiner unless generalization of the disease to the lips or other sites of the animal had occurred. In another experiment we exposed examiners to the heads of the pigs and to the remaining parts of the pigs; the parts not sampled were held in a plastic bag. Although lesions were present on the feet as well as on the tongue and snout, more virus was recovered from the noses of those examining the head. These preliminary results suggest that the source of airborne virus might be some part of the upper respiratory tract. No virus has been recovered from people examining infected mice or guinea-pigs. Gibbs (1931) found that guinea-pigs did not readily transmit virus to others in the same cage, but that hedgehogs did. Edwards (1934) recovered virus from the breath of infected hedgehogs and demonstrated multiplication of FMD virus in the nasal mucous membranes. He failed to recover virus from the breath of infected guinea-pigs. It may be that examination of infected hedgehogs might lead to recovery of virus from the examiner's nose. At the laboratory bench, virus ($10^{2 \cdot 9}$ ID50) was recovered from the nose of one of us immediately after the collection of supernatant fluids from BHK21 cells in Roux bottles infected with C Noville virus.

Inhalation of such large amounts of virus over a period has not resulted in any clinical signs of foot-and-mouth disease in Institute workers who have handled animals infected with foot-and-mouth disease. The results of our antibody tests revealed that one of 10 sera examined showed some antibody to type C, and there was no conversion to positive as a result of exposure. This is not surprising as FMD in man is rare. In reported cases of FMD in man (Vetterlein, 1954; Heinig & Neumerkel, 1964; Pilz & Garbe, 1965; Armstrong, Davie & Hedger, 1967; Eissner, Böhm & Jülich, 1967; Suhr Rasmussen, 1968) the source of infection was attributed to drinking infected milk, accidental self-inoculation or to handling infected animals while the skin was damaged by cuts, manicure or by dermatitis. It is likely that in some instances the patients would have inhaled virus in reasonable amounts and infection could also be ascribed to this route. Of the 37 cases reported 23 were due to type O, 13 to type C and 1 to type A virus. In a serological survey of workers exposed to FMD virus, Suhr Rasmussen (1968) and Wisniewski &

Jankowska (1968) found that low titre antibodies to type O were the most prevalent followed by type C. In this connexion it is interesting to note that the highest yields of airborne virus were recovered from animals infected with O_1 or C Noville strains of virus (Donaldson, *et al.* 1970).

The inhalation, retention, clearance and dispersal of particles in the nasal region has been extensively investigated by the use of physical and chemical substances as well as bacteria and viruses (Buckland & Tyrrell, 1964; Buckland, Bynoe & Tyrrell, 1965; Druett, 1967). In many of these investigations subjects were exposed to artificially generated aerosols or materials such as spores or viruses were placed in the nostrils. The materials varied in their adhesive properties; some, such as pollen or fungal spores, showed greater penetration, whereas others adhered to the nasal hairs or attached themselves to cells. With rhino- and Coxsackie-viruses, infection of cells together with virus multiplication and production of nasal secretion took place. Our results were obtained by exposure of subjects to a naturally generated aerosol, measurements of which were made to determine virus recovery and particle size. In addition FMD virus is similar in certain properties to rhinoviruses, e.g. pH lability and size. Unlike rhinoviruses, FMD virus did not multiply in the nose but may have attached to nasal hairs and cells. Our results may therefore represent some of the aspects of initiation of infection by respiratory viruses before multiplication has occurred and fill a gap between the results of Buckland & Tyrrell (1964) with bacterial spores and bacteriophage and those of Buckland *et al.* (1965) and Gerone *et al.* (1966) with Coxsackie virus A21.

On exposure to infected animals the nose rapidly took up FMD virus, but the virus concentration did not increase after 5 min. while the subject remained in the particular environment. When the subject moved to a higher concentration of virus, e.g. from cattle boxes to pig boxes, the amount of virus in the nose increased until an equilibrium was reached. When he moved out of the infected environment, the amount in the nose decreased at the rate of 1·1 to 1·8 log. units in an hour. This was slower than the fall in titre recorded for *Bacillus mycoides* spores, namely 1·4 and 2·6 log. units in 40 min. (Buckland & Tyrrell, 1964). It may be that FMD virus attached better to cells than the spores, and this suggests that a longer period is available for respiratory viruses to attach. In addition with FMD virus clearance may not be complete, since even after 10 nose blows virus was recovered in nasal swabs. Activities such as nose blowing or snorting may represent another chance for virus to attach to cells. The amount of FMD virus recovered from nose blows was the same as from nasal swabs, whereas volunteers infected with Coxsackie virus A21 shed more virus in the nose blows than in the nasal washings. This difference may be due to the nose blow bringing forward secretion from an actively multiplying site of A21 virus. In our experiments on an average seven times more nasal secretion was found in a nose blow than in a nasal swab. Since the virus titres were the same, this probably means that a certain proportion of virus is attached to the cells and cannot be removed by the nose blows. The amounts of virus recovered in saliva and throat swabs were less than the amounts recovered in nasal swabs as demonstrated by Buckland & Tyrrell (1964). Sneezes were considered to be good dispersers of virus and in our experiments gave good recovery,

although it must be pointed out that we measured only the amounts attached to the wall of the plastic bag and not the enclosed air. We also recovered virus from breathing and talking on two occasions, although these activities are not considered good dispersers of bacteria or viruses. Tyrrell (1967) has pointed out that in respiratory infections virus may be spread by individuals who excrete particularly high concentrations of virus. In our experiments, despite comparable exposure, there was variation between subjects in the amount of virus in nasal and other secretions and in the rate of clearance. It may be that this variation in anatomical and physiological factors may influence the chance of infection and spread of respiratory viruses.

No experiments have been done to see whether FMD can be transferred from one animal to another through exhalation of virus by man. However, when people who had been examining infected animals talked to colleagues for 4 min., virus was subsequently recovered from the nose of one of the colleagues, although the virus could have come from the clothing of the examiners as well as from the exhaled air. This is a further indication of how rapidly a respiratory virus may be transmitted. Industrial, surgical or paper masks were not effective in protecting the nose from inhalation of virus. It could be argued that only the smaller particles were penetrating; on the other hand virus was still recovered from the nasal passages, where only particles greater than 6 μ are retained (May, 1966) so it would appear that a proportion of the larger particles were entering the nose. To assume therefore that such masks afford protection against inhaling or exhaling infective virus is false; the only effective method of protection would be provided by respirators capable of trapping large and small particles. To avoid transferring FMD virus it would be advisable to allow natural clearance of virus from the nose. Virus swallowed would be rapidly inactivated by the low pH in the stomach.

Our results emphasize that the respiratory tract of man or animal is a most effective device for sampling aerosols, and details of the amounts recovered may be used to determine whether multiplication of virus has occurred either in man exposed to respiratory viruses or in animals exposed to FMD.

We should like to thank our colleagues, who subjected themselves so readily to the various activities. Mr E. H. Knight kindly drew our attention to the unpublished work of the late Dr J. T. Edwards. We are grateful to Miss C. D. Mills, Miss J. Piper, Miss J. Slater, N. H. Cheale and D. R. Taylor for technical assistance.

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J. Hyg., Camb. (1970), **68**, 575
With 1 *plate*
Printed in Great Britain

The late detection of respiratory syncytial virus in cells of respiratory tract by immunofluorescence

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SUMMARY

Paired nasopharyngeal secretions were studied in 27 infants infected with respiratory syncytial virus, one taken at onset of illness and one about 7 days later. Both specimens were examined by immunofluorescence and tissue culture for respiratory syncytial virus. In 25 out of 27 (93 %) specific fluorescence was still present in cells of the convalescent specimen but was much duller. Virus was difficult to isolate in convalescent specimens; only 8 out of 27 (26 %) proved to be positive. Eight single secretions which were taken late in a respiratory illness were also shown to have this altered fluorescence with absence of virus isolation. Preliminary experiments using antihuman globulin suggest that the findings may be due to the attachment of local secretory antibody to the cells causing 'blocking' of staining reaction.

INTRODUCTION

Cells aspirated in nasopharyngeal secretions from the respiratory tracts of young infants were first used for the detection of respiratory syncytial (R.S.) virus by immunofluorescence in 1968 and the technique has since become a standard method for the identification of the virus in this laboratory (McQuillin & Gardner, 1968; Gardner & McQuillin, 1968). The secretions taken for the rapid diagnosis of R.S. virus infections were also used for the investigation of local neutralizing antibodies to R.S. virus in the respiratory tract; it is believed that in many acute respiratory virus infections, local antibodies bear a closer relationship to protection than do humoral antibodies (Smith, Purcell, Bellanti & Chanock, 1966; Kim *et al.* 1969). For the study of the production of local antibody in the respiratory tract, two specimens of secretion were taken at approximately 7-day intervals during the course of an acute illness. It was decided to investigate whether the development of local antibody produced any change in the fluorescent staining of virus antigen in R.S. virus-infected respiratory cells. Among other aims of this investigation were: the estimation of the length of time that virus antigen could still be detected in nasopharyngeal secretions; the determination as far as possible of the length of time that these secretions remain infective; and the study of the relationship between infectivity and fluorescence. This approach was used as a result of previous observations that R.S. virus-infected cells in nasopharyngeal

secretions of children examined late in their illness showed a duller fluorescence; this appearance often coincided with a delay in the time taken to isolate the causal virus on tissue culture.

MATERIALS AND METHODS

Paired nasopharyngeal secretions were taken from 27 infants; the first specimen, called the acute specimen, was taken within 48 hr. of onset of illness and the second specimen, called the convalescent specimen, approximately 7 days later. The exact interval between the two secretions is shown in Table 1, which also shows the age of the children. The method for collection of secretions has been described in previous publications (Gardner & McQuillin, 1968; Sturdy, McQuillin & Gardner, 1969). The 27 children were all known to be infected with R.S. virus by the previous examination of their acute secretions by immunofluorescence and virus culture; the convalescent specimens of secretion were examined by the same methods for R.S. virus.

In addition, during the course of a 2-year survey of acute respiratory infections of childhood, in which the fluorescent antibody technique was used, there were eight children from whom no virus was isolated, but specific fluorescence for R.S. virus was observed in cells of their nasopharyngeal secretions. Secretions from these children were further investigated and formed part of this study.

The tissue culture and immunofluorescent techniques employed were identical with those described elsewhere (Gardner & McQuillin, 1968; Sturdy, McQuillin & Gardner, 1969). In addition, a number of nasopharyngeal secretions were stained with fluorescein-labelled antihuman globulin by methods which have also been described (Gardner, McQuillin & Court, 1970).

Extra slides were always prepared from every specimen of nasopharyngeal secretion which came to the laboratory; all slides were fixed in acetone and stored at $-40°$ C. These stored preparations could be used for various purposes, including research investigations such as described here.

RESULTS

Table 1 gives the detailed results of the examination of the paired secretions for both immunofluorescence and isolation of virus. The relationship between the isolation of R.S. virus and the detection of its presence by immunofluorescence in the 27 second secretions is shown in Table 2. In 18 out of 27 secretions, the results for the fluorescent antibody technique were positive, but R.S. virus was not isolated, and in only seven infants did isolation of virus confirm the positive fluorescence. The acute secretions of all these patients, when stained by the fluorescent antibody technique, showed the classical appearance of strongly fluorescent intracellular R.S. virus and this is illustrated in Pl. 1, fig. 1. Fig. 2 shows the much duller, but nevertheless specific, fluorescence observed in 25 out of the 27 convalescent secretions.

The cells in the single secretions of eight children from whom virus was not isolated showed specific fluorescence for R.S. virus. During this period, there were 224 additional R.S. virus infections diagnosed by immunofluorescence, all con-

Table 1. *Summary of examination of paired secretions from 27 patients with R.S. virus infection*

Case no.	Name	Age	Specimen of secretion	Date received	No. of days between specimen	F.A. result for R.S.V.	Isolation of R.S. virus
1	S.S.	5 months	Acute	30. x. 68	7	+	+
			Convalescent	6. xi. 68		+	−
2	S.C.	10 weeks	Acute	4. xi. 68	7	+	+
			Convalescent	11. xi. 68		+	−
3	C.W.	7 weeks	Acute	4. i. 69	6	+	+
			Convalescent	10. i. 69		+	−
4	R.S.	10 weeks	Acute	21. x. 69	6	+	+
			Convalescent	27. x. 69		+	−
5	S.W.	10 months	Acute	3. xi. 69	4	+	+
			Convalescent	7. xi. 69		+	−
6	H.S.	3 months	Acute	11. xi. 69	6	+	+
			Convalescent	17. xi. 69		+	−
7	W.I.	12 weeks	Acute	2. xii. 69	7	+	+
			Convalescent	9. xii. 69		+	+
8	A.L.	6 weeks	Acute	9. xii. 69	7	+	+
			Convalescent	16. xii. 69		+	−
9	D.H.	13 months	Acute	11. xii. 69	7	+	+
			Convalescent	18. xii. 69		+	−
10	C.T.	6 months	Acute	12. xii. 69	7	+	+
			Convalescent	19. xii. 69		+	−
11	K.R.	6 weeks	Acute	13. xii. 69	9	+	+
			Convalescent	22. xii. 69		+	+
12	J.B.	7 weeks	Acute	15. xii. 69	8	+	+
			Convalescent	23. xii. 69		+	+
13	D.B.	11 weeks	Acute	22. xii. 69	7	+	+
			Convalescent	29. xii. 69		+	−
14	M.E.	5 months	Acute	24. xii. 69	9	+	+
			Convalescent	2. i. 70		−	−
15	A.L.	9 months	Acute	9. i. 70	7	+	+
			Convalescent	16. i. 70		+	−
16	M.H.	7 weeks	Acute	9. i. 70	7	+	+
			Convalescent	16. i. 70		+	−
17	L.G.	2 months	Acute	10. i. 70	6	+	+
			Convalescent	16. i. 70		+	+
18	G.H.	8 months	Acute	19. i. 70	7	+	+
			Convalescent	26. i. 70		+	−
19	R.D.	8 months	Acute	22. i. 70	7	+	+
			Convalescent	29. i. 70		+	−
20	G.W.	10 weeks	Acute	22. x. 69	5	+	+
			Convalescent	27. x. 69		+	+
21	S.B.	10 weeks	Acute	22. i. 70	7	+	+
			Convalescent	29. i. 70		−	−
22	S.A.	2 months	Acute	22. i. 70	6	+	+
			Convalescent	28. i. 70		+	−
23	D.C.	2 weeks	Acute	6. ii. 70	6	+	+
			Convalescent	12. ii. 70		+	+
24	K.H.	4 months	Acute	9. ii. 70	7	+	+
			Convalescent	16. ii. 70		+	+
25	M.C.	4 months	Acute	13. ii. 70	6	+	+
			Convalescent	19. ii. 70		+	−
26	S.S.	4 months	Acute	23. ii. 70	7	+	+
			Convalescent	2. iii. 70		+	−
27	C.A.	5 weeks	Acute	15. iii. 70	11	+	+
			Convalescent	26. iii. 70		+	−

firmed by isolation of virus. The positive cells in all eight secretions showed a duller fluorescence identical with that observed in those 27 secretions taken in the convalescent phase of the illness. The picture they presented is illustrated in Pl. 1, fig. 3. The study of the clinical histories of these children showed that they had all been ill for a minimum of 5 days and four of them for a fortnight before a secretion was taken for examination. These details are shown in Table 3.

Table 2. *The relationship between isolation technique and fluorescent antibody technique in identifying R.S. virus in 27 second secretions*

R.S. virus isolated. F.A. positive	R.S. virus not isolated. F.A. positive	R.S. virus isolated. F.A. negative	R.S. virus not isolated. F.A. negative
7	18	0	2

Table 3. *Findings in single secretions from children: immunofluorescence positive, culture negative for R.S. virus*

Case no.	Name	Age when specimen taken	Length of illness before specimen taken (days)
28	S.T.	6 weeks	14
29	A.B.	4 months	14
30	C.P.	22 months	7
31	P.K.	4 months	5
32	D.M.	15 months	14
33	B.K.	18 days	6
34	D.F.	28 days	14
35	D.W.	8 weeks	7

The changes observed by immunofluorescence in the staining properties of the cells in the convalescent secretions coincided in a number of instances with an increase of local neutralizing antibody which would account for the increased difficulty of isolation of virus. It was therefore decided to examine three sets of paired secretions by using fluorescein-labelled antihuman globulin in parallel with the examination by the original method of indirect fluorescent antibody staining for R.S. virus. Two single specimens of secretion from which we failed to isolate R.S. virus were treated in the same way. In both these specimens a large number of cells were observed to have been stained with the fluorescein-labelled antihuman globulin. An example of such a cell is illustrated in Pl. 1, fig. 4. In the three paired secretions, there was an increase in fluorescence in the convalescent secretion as compared with the acute, but it was difficult to put this observation on a firm quantitative basis. The number of cells fluorescing with antihuman globulin did not always appear equal to the number of cells showing abnormal staining with R.S. virus antiserum and anti-rabbit globulin; there was also some variation in the nature of the fluorescence.

DISCUSSION

The results showed that specific fluorescence for R.S. virus remained in the cells of the nasopharyngeal secretion for 25 out of 27 patients (93 %) for at least 7 days after the onset of acute symptoms, but in the second specimen the fluorescence was much duller. Isolation in the convalescent specimen was only effective in seven out of 27 (26 %). The identical picture seen in eight specimens taken late in the respiratory illness from which virus was not isolated suggests that the failure to isolate virus in these cases was not due to false positive fluorescence but to the late stage in the illness when the specimen was taken. There are three possible explanations for this failure. One explanation may be that the 'blocking' of virus and decrease of infectivity may result from the development of the patient's own neutralizing antibody; another, that the change in fluorescent appearance, coupled with the failure to isolate, is due to antigen persisting in the cells which, although it can still be detected by immunofluorescence, has undergone some deterioration and is no longer infective. A third simple explanation could be faulty isolation techniques, but the successful isolation of virus from all acute specimens of the 27 paired secretions, in addition to the failure to isolate from the eight other patients, with single secretions, where there was delay between onset of illness and collection of specimen, appears to rule out this possibility.

Scott & Gardner (1970) have shown that a number of these paired secretions developed neutralizing antibody which coincided with the change in fluorescent appearance and loss of infectivity. Kim and her colleagues in 1969 have also shown that neutralizing antibodies to R.S. virus develop in nasal secretions during the course of a respiratory infection. The result of the preliminary and limited investigation performed on these secretions using antihuman globulin suggests, too, that during the course of the illness human globulin becomes attached to nasopharyngeal cells. We have no direct evidence, however, that the antibody which may be coating cells in the nasopharynx is R.S. virus antibody. We believe that it could be because of the coinciding rise of neutralizing antibody in a number of paired secretions and the demonstration of human globulin on cells in convalescent specimens. The development of this altered fluorescence, if due to 'blocking' by human globulin and the coinciding rise of neutralizing antibody, gives support to this hypothesis. The attachment of antibody *in vivo* to virus-infected cells might occur, provided that the membranes of the infected cells have been altered by the invading virus or virus protruding through the cell membrane. R.S. virus has been classified with the myxoviruses which have an intimate connexion with the surface of their host cell.

Scott & Gardner (1970) have also suggested, as a result of some hitherto unpublished experiments, that IgA is the predominant fraction of globulin present in the nasal secretions of all these infants. The examination of cells from more secretions with specific fluorescein-labelled anti-IgA will demonstrate whether it is this antibody which is attaching itself to infected cells.

If it can be shown that this 'blocking' of fluorescence observed in cells of convalescent secretions is due to development of neutralizing antibodies binding to

infected cells, it will provide evidence that this mechanism is a factor in limiting the length of time that a patient remains infective. The possibility of deterioration of antigen must be explored further should it not be proven that antibody attaches itself to the cells. This seems less likely, as the cells appear to be intact and in as good condition as those observed in the acute specimens of secretion.

Antibody coating cells in the respiratory tract is unlikely to be IgG. If maternal IgG penetrated to the respiratory tract in acute infections, the cells in the acute specimen of secretion should show this 'blocking' effect. The use of antisera to the individual fractions of globulin will throw light on this very important topic.

We are indebted to Professor Court and his colleagues in the Department of Child Health for their help in supplying clinical material. We are also grateful to the Medical Research Council for its support.

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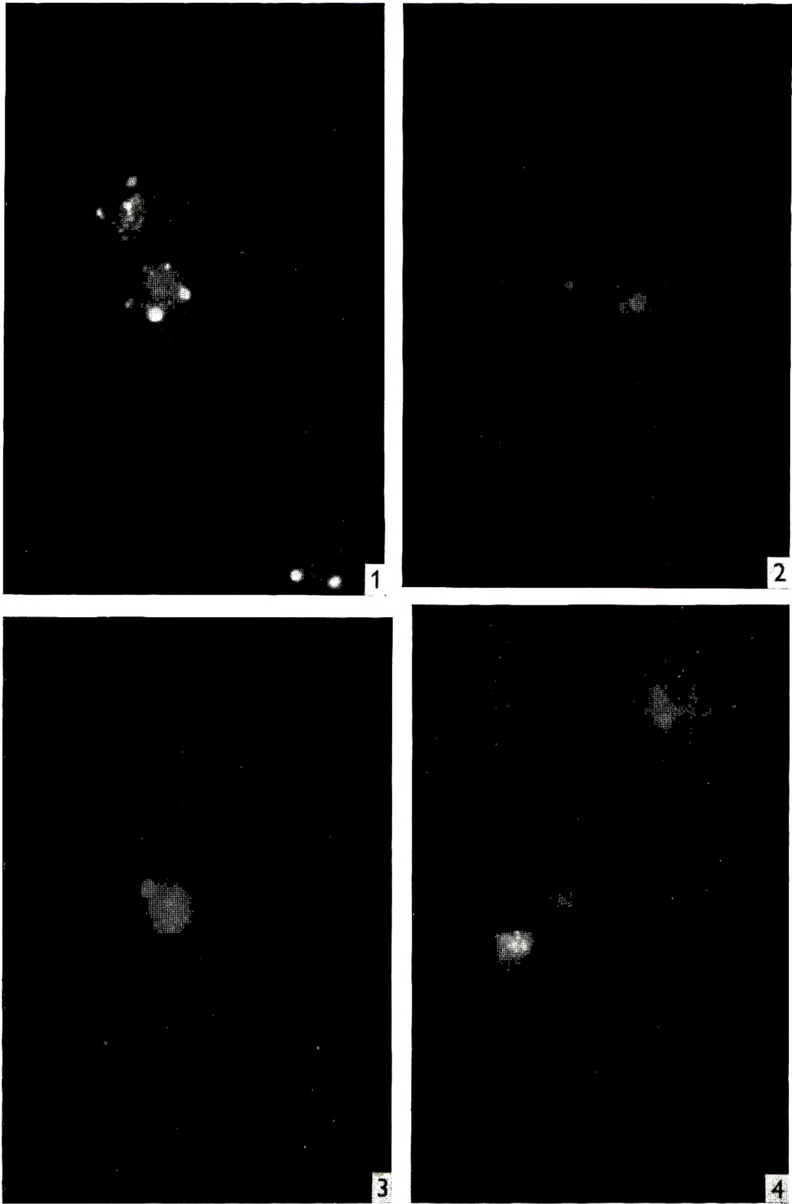
EXPLANATION OF PLATE

Fig. 1. Cells in a nasopharyngeal secretion taken at the onset of bronchiolitis and stained by the indirect fluorescent antibody technique for R.S. virus. Magnification × 1200.

Fig. 2. A cell in nasopharyngeal secretion taken 7 days after first secretion and stained by the indirect fluorescent antibody technique for R.S. virus; the duller fluorescence is demonstrated. Magnification × 1200.

Fig. 3. A cell in a nasopharyngeal secretion taken late in bronchiolitis and stained by the indirect fluorescent antibody technique for R.S. virus; the duller fluorescence is demonstrated. Magnification × 1200.

Fig. 4. A cell in convalescent nasopharyngeal secretion stained by fluorescein-labelled antihuman globulin; the attachment of human globulin to the cell is demonstrated. Magnification × 1200.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J. Hyg., Camb. (1970), **68**, 581
Printed in Great Britain

Respiratory syncytial virus neutralizing activity in nasopharyngeal secretions

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(*Received* 2 *June* 1970)

SUMMARY

Nasopharyngeal secretions were taken during the acute phase of illness from 30 children admitted to hospital with lower respiratory tract infections. The presence of neutralizing activity in secretions taken at the onset of illness was demonstrated in 11 out of 15 patients (73 %) with bronchiolitis caused by R.S. virus, as compared with 4 out of 9 patients (44 %) with R.S. virus infections other than bronchiolitis, and 1 out of 6 without R.S. virus infection. Second secretions were taken 7 days later from 10 of the children with R.S. virus infection. Eight of these paired secretions showed an increase of neutralizing activity against R.S. virus. It is suggested that the neutralizing activity, found in secretions taken at the onset of illness, may be a result of previous infection with R.S. virus.

INTRODUCTION

The possibility that nasal secretions of patients recovering from influenza contain antibodies which inhibit influenza A virus was first suggested by Francis (1940). Recent studies which have involved giving parainfluenza type I (Smith, Purcell, Bellanti & Chanock, 1966) and rhinovirus type 13 (Perkins *et al.* 1969) to volunteers have shown that local secretory antibody is more important than serum antibody in protection against subsequent infections. Since respiratory syncytial (R.S.) virus has many properties in common with myxoviruses, and because of its overwhelming importance in both lower respiratory tract infections and respiratory deaths in childhood (Chanock *et al.* 1961; Aherne *et al.* 1970), it is surprising to find, as yet, few reports of the local production of antibody stimulated by infection with this virus (Kim *et al.* 1969*a*). This investigation was initiated to study local antibody response in nasopharyngeal secretions in children with R.S. virus infections. Another aim of the study was to discover whether neutralizing antibody occurred in nasal secretions of patients with R.S. virus infection and if it did, whether children with R.S. virus infection develop increasing antibody titres as part of a local response.

We also wished to investigate the possibility that a sensitivity reaction may be associated with R.S. virus bronchiolitis, as has been suggested by some workers (Gardner, McQuillin & Court, 1970; Chanock *et al.* 1968). Both type 1 and type 3 allergic reactions (Gell & Coombs, 1968) have been postulated to explain the patho-

genesis of bronchiolitis, and if antibody could be found in the secretions during the acute phase of an R.S. virus infection, then this would tend to support a type 1 allergic reaction.

MATERIALS AND METHODS

Patients

The patients included in this study were children between 4 weeks and 20 months of age who were admitted to hospital with acute lower respiratory tract infections. The categories of clinical illness used have been previously defined (Gardner *et al.* 1960). In most infants with R.S. virus infection the clinical diagnosis was bronchiolitis. A nasopharyngeal secretion was obtained from the patient within 24 hr. of admission to hospital, and, whenever possible, a second secretion was obtained after an interval of 7 days.

Tissue culture techniques

The growth, maintenance, and overlay media used in the plaque technique described below were prepared in 100 ml. amounts. The growth medium for HEp 2 cells consisted of Eagle's minimum essential medium containing 10 ml. calf serum, 50 mg. penicillin, 25 mg. streptomycin, 12·5 mg. nystatin, and 12·5 mg. neomycin. The maintenance medium for HEp 2 cells consisted of medium 199 containing 2 ml. embryo calf serum, 0·6 ml. 0·25 % glutamine, and the same concentration of antibiotics as in the growth medium. The overlay medium consisted of Eagle's minimum essential medium containing 10 ml. heat inactivated embryo calf serum, 0·6 ml. 0·25 % glutamine, 0·75 g. methyl cellulose, and the same concentrations of antibiotics as in the growth medium with the exclusion of neomycin.

Collection and preparation of specimens

The method of collection of secretions has been fully described elsewhere (Sturdy, McQuillin & Gardner, 1969). The secretion was partially purified by initial centrifugation at 1000 rev./min. for 10 min. at 4° C. in order to deposit the cells. The supernatant was shaken with glass beads in a Griffith's shaker at 4° C. for 1 hr. and subsequently centrifuged at 3000 rev./min. for 2 hr. at 4° C. The supernatant was separated from the mucus and cell debris, and stored at $-20°$ C. until needed for tests.

Estimation of neutralizing activity

The neutralizing activity of the nasopharyngeal secretion was measured by a modification of the R.S. virus plaque reduction technique, first described by Coates, Alling & Chanock in 1966. The protein content of the secretions was measured by a microtechnique based on the original method of Lowry, Rosebrough, Farr & Randall in 1951, and the neutralizing titres of the nasopharyngeal secretion were adjusted to a protein level of 10 mg./ml.

HEp 2 cells were seeded in disposable tissue culture plates, 35 mm. in diameter and 10 mm. in depth, at a concentration of 5×10^5 cells per plate, in 3 ml. of

growth medium. After incubation for 24–30 hr. in a humidified atmosphere of 5 % CO_2 in air, the cells formed a monolayer.

A stock of the Long strain of R.S. virus was grown in HEp 2 cells and stored in 1 ml. ampoules in liquid nitrogen. The TCD 50 of the virus stock, as estimated in HEp 2 tissue culture tubes was $10^{-4\cdot5}$. This corresponded to a titre of 10^6 p.f.u./ml., as estimated by the plaque technique.

The virus suspension was diluted in maintenance medium to give a final concentration of 250 p.f.u./ml. when inoculated on the tissue culture plates of HEp 2 cells. The secretions were inactivated at 56° C. for 30 min. and diluted in maintenance medium, in twofold dilutions starting at 1/4. Equal volumes of virus suspension and diluted secretion were mixed; after 1 hr. at room temperature, 0·2 ml. of each virus–secretion mixture was inoculated on two HEp 2 plate cultures, which were incubated at 37° C. for 2 hr. in a humidified atmosphere containing 5 % CO_2 in air. In addition, two plates containing 0·2 ml. diluent which acted as negative controls, and a virus titration, which consisted of three plates containing 0·2 ml. of an equal volume of virus suspension and diluent, were similarly incubated.

After the virus had been allowed to adsorb onto the cells, they were washed with Hanks's balanced salt solution, and 4 ml. amounts of the overlay medium were added to each plate. The plates were reincubated at 37° C. in a humidified atmosphere of 5 % CO_2 in air. Four days later the cells of one virus titration plate were fixed with 10 % formalin and stained with haematoxylin and eosin. According to the stage of development of the plaques, the cells in the remaining plates were either fixed and stained immediately or reincubated for a further 24 hr. The plaques were counted, using a Vickers Sterimag II microscope (magnification × 20) and the titre of neutralizing activity was calculated as the dilution of the secretions producing 60 % or more reduction in the plaque count.

Owing to the very small volume of secretions usually available, it was necessary to dilute some of the secretions in saline, before the estimation of neutralizing activity and protein content.

The cells in the nasopharyngeal secretions were investigated for the presence of R.S. virus by the fluorescent antibody technique (Gardner & McQuillin, 1968) as part of the routine diagnostic investigation on a patient; secretions were also inoculated on HEp 2, Bristol HeLa, monkey kidney and W.I. 38 cell lines, to confirm the presence of any virus (Sturdy *et al.* 1969).

RESULTS

Nasopharyngeal secretions taken from 30 patients within 24 hr. of admission to hospital were examined (Tables 1 and 2). Second secretions taken 7 days after admission from 10 of these 30 patients, were also examined (Table 2). In this group of 10 patients from whom paired secretions were examined, eight developed a rise in titre of nasal secretory neutralizing activity (adjusted to 10 mg./ml. protein). In four of these eight patients the rise in neutralizing activity was fourfold or greater. Furthermore, the presence of neutralizing activity at a level of

1/10 or greater in the first secretions of the 10 patients (Table 2), appeared to give some indication of the subsequent response. Two of the three patients with a titre of neutralizing activity of 1/10 or greater in their first secretion did not show any increase in neutralizing activity in their second secretion.

Table 1. *Neutralizing activity in acute nasopharyngeal secretions of children with lower respiratory tract infections*

Patient no.	Diagnosis	Age	Isolation of virus in nasopharyngeal secretion	R.S. virus plaque reduction titre	Protein content (mg./ml.)	R.S. virus plaque reduction titre adjusted to 10 mg./ml.
1	Bronchiolitis	5 months	R.S. virus	1/4	3·2	1/13
2	Bronchiolitis	3 months	R.S. virus	1/4	11·4	1/4
3	Bronchiolitis	4 months	R.S. virus	1/4	4·9	1/8
4	Bronchiolitis	7 weeks	R.S. virus	1/16	1·9	1/84
5	Bronchiolitis	2 months	R.S. virus	1/32	8·1	1/40
6	Bronchiolitis	8 weeks	R.S. virus	1/32	10·0	1/32
7	Bronchiolitis	10 months	R.S. virus	< 1/4*	8·5	1/2
8	Bronchiolitis	7 weeks	R.S. virus	< 1/4	6·2	1/3
9	Bronchiolitis	5 weeks	R.S. virus	1/4	11·3	1/4
10	Bronchiolitis	9 months	Adenovirus	< 1/4	11·6	1/2
11	Bronchiolitis	4 weeks	Negative	< 1/4	17·1	1/1
12	Pneumonia	7 months	R.S. virus	< 1/4	9·2	1/2
13	Pneumonia	4 months	R.S. virus	< 1/4	11·2	1/2
14	Pneumonia	4 months	R.S. virus	< 1/4	6·7	1/3
15	Pneumonia	3 months	Parainfluenza III	< 1/4	9·7	1/2
16	Bronchitis	5 months	R.S. virus	< 1/4	6·4	1/3
17	Bronchitis	11 months	R.S. virus	< 1/4	7·2	1/3
18	Croup	20 months	Negative	< 1/4	5·2	1/4
19	Croup	12 months	Parainfluenza I	1/4	16·9	1/2
20	Croup	4 months	Negative	< 1/4	6·2	1/3

* < 1/4 is considered as 1/2 for adjusting R.S. virus plaque reduction titres to 10 mg./ml. of protein.

In 11 of the 15 patients (73%) admitted with bronchiolitis, and from whom R.S. virus was isolated, a neutralizing titre of 1/4 or greater was found in their first secretion (Tables 1 and 2). However, in the nine patients with illnesses other than bronchiolitis, and from whom R.S. virus was isolated, only four (44%) had a neutralizing titre of 1/4 or greater in their first specimen. Furthermore, only one of the six patients, from whom R.S. virus was not isolated, possessed neutralizing activity at a titre of 1/4 or greater in the first secretion.

The development of a rise in titre of secretory neutralizing activity, or the presence in the secretions of a high neutralizing titre during the acute phase of illness did not appear to be related to age.

Table 2. *Neutralizing activity in paired nasopharyngeal secretions of children with lower respiratory tract infections*

Naso-pharyngeal secretion	Patient no.	Diagnosis	Age	No. of days between secretion	Virus diagnosis	Reciprocal R.S. virus plaque reduction titre	Protein content (mg./ml.)	Reciprocal of R.S. virus plaque reduction titre adjusted to 10 mg./ml.
First	21	Bronchiolitis	3 months	6	R.S. virus	8	7·8	10
Second					Negative	4	15·5	3
First	22	Bronchiolitis	5 months	7	R.S. virus	< 4*	5·3	4
Second					R.S. virus	8	6·3	13
First	23	Bronchiolitis	10 weeks	7	R.S. virus	4	10·4	4
Second					Negative	32	3·2	100
First	24	Bronchiolitis	10 weeks	7	R.S. virus	< 4	11·7	2
Second					Negative	8	11·9	7
First	25	Bronchiolitis	12 weeks	7	R.S. virus	< 4	1·1	18
Second					R.S. virus	8	6·1	13
First	26	Bronchiolitis	4 months	6	R.S. virus	< 4	16·3	1
Second					R.S. virus	8	6·9	12
First	27	Bronchitis	10 months	4	R.S. virus	4	9·4	4
Second					R.S. virus	16	9·5	17
First	28	Bronchitis	13 months	7	R.S. virus	4	6·8	6
Second					R.S. virus	8	2·2	36
First	29	Bronchitis	6 weeks	7	R.S. virus	8	13·2	6
Second					Negative	< 4	0·9	22
First	30	Bronchitis	8 months	7	R.S. virus	< 4	2·1	10
Second					R.S. virus	8	3·6	22

* < 1/4 is considered as 1/2 for adjusting R.S. virus plaque reduction titres to 10 mg./ml. of protein.

DISCUSSION

It is now generally accepted that R.S. virus is the most important respiratory pathogen of early childhood (Chanock *et al.* 1961; Aherne *et al.* 1970). In lower respiratory tract infections it has been found that the majority of infants affected are less than 6 months of age (Elderkin, Gardner, Turk & White, 1965). At this age, maternally derived serum antibody (IgG) is present, but it does not appear to exert any protective effect. Furthermore, recent studies involving an inactivated R.S. virus vaccine showed that development of high levels of serum antibody did not prevent subsequent natural infection. Not only did vaccination fail to produce immunity, but it actually increased the severity of illness in patients who were subsequently naturally infected (Chin *et al.* 1969; Fulginiti *et al.* 1969; Kapikian *et al.* 1969; Kim *et al.* 1969*b*).

Since serum antibody does not appear to play a major protective role in R.S. virus infections, the present study has been directed at the role of local antibody present in the respiratory tract. The development of R.S. virus neutralizing activity in nasal secretions of children with R.S. virus lower respiratory tract infections has been recently described by Kim and her colleagues in 1969 and the presence of neutralizing activity in the first secretions was also demonstrated. However, these workers did not attempt to classify the R.S. virus infections into clinical categories.

The results obtained in the present study are in agreement with those obtained by Kim and her colleagues, but it was further found that a high titre of neutralizing activity was more prevalent, during the acute stage of illness, in patients with R.S. virus bronchiolitis than in patients with other types of R.S. virus infection. Although Kim and her colleagues often found neutralizing activity present in the first secretions, taken early in illness, they explained this in terms of the speed of development of nasal secretory antibody. Another possible explanation is that the neutralizing activity was already present, due to previous infection, or its rapid rise was due to previous sensitization with R.S. virus (Gardner, McQuillin & Court, 1970).

Although patients admitted to hospital with bronchiolitis often had histories of upper respiratory tract infections, these were of short duration and all were admitted within 48 hr. of the onset of acute symptoms. Speed of development of neutralizing titre in paired secretions (Table 2) which was always within 7 days, tends to support the hypothesis of a previous exposure and possible sensitization with R.S. virus antigen.

It was found that the calculation of neutralizing titre was noticeably affected by the percentage reduction of plaque count used in the calculation. If, instead of using the purely arbitrary figure of 60 % reduction, a reduction of 50 % was used in the calculation, the number of patients with a fourfold rise in neutralizing titre was increased from 4 to 5 (Table 2).

The exact nature of the neutralizing activity demonstrated is presumably antibody, and it is hoped that this impression may be confirmed by examining a larger series of patients. It is improbable that the neutralizing activity is due to

interferon, as the rises in neutralizing titres occur at a time when antibody and not interferon would be increasing. Further studies are being undertaken to confirm that the nature of neutralizing activity is antibody. If this is so, it is unlikely to be maternal IgG, as this would not be consistent with the rising titres found in the paired secretions. Furthermore we have found, in a preliminary investigation using immunoelectrophoresis and quantitative Hyland Immuno-plates (Fahey & McKelvey, 1965), that the only type of immunoglobulin consistently present at a substantial level in the respiratory tract is IgA.

An investigation of 27 paired nasopharyngeal secretions by immunofluorescence has also been undertaken (Gardner McQuillin & McGuckin, 1970). It was found that the cells in the second secretion appeared duller on staining, as if 'blocked'; virus isolation from these secretions often proved unsuccessful. These results tend to support the conclusion that the neutralizing activity in the nasopharyngeal secretions may be due to antibody.

We are indebted to Professor S.D.M. Court and his colleagues, of the Department of Child Health for their help in supplying the clinical material. We are also grateful to the Medical Research Council for its support.

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Printed in Great Britain

Tuberculous morbidity in Swiss albino mice immunized with BCG*

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(*Received* 8 *June* 1970)

SUMMARY

Study was made of the effect of BCG vaccination on the establishment of experimental tuberculous infection in mice. To this end, mice were infected with small quantities of virulent human bacilli administered by the respiratory route. The quantity of bacilli present for inhalation was so controlled as to permit only a finite percentage of unvaccinated animals to become infected. The relative proportion of mice infected by inhaling these small numbers of virulent bacilli was not found to be lowered by prior BCG vaccination. Thus, pulmonary tissue of immunized mice was not able to prevent the initial lodgement of virulent tubercle bacilli. The growth of virulent organisms in infected lungs, however, was significantly retarded by prior vaccination.

INTRODUCTION

BCG vaccination increases resistance to tuberculosis both in experimental animals and in man. In experimental animals this enhanced resistance is manifested as an alteration of the progress of disease or as a delay in the onset of tuberculous mortality (Dubos, Pierce & Schaefer, 1953). Contrariwise in man, vaccination decreases the primary incidence of tuberculous infection as well (Medical Research Council, 1963). Thus, human pulmonary tissue appears capable of acquiring greater immunity to tuberculosis than does similar tissue from lower mammals.

The evidence bearing on this question, however, is largely of an indirect nature. It has been found, for example, that similar numbers of bacilli are present in lungs of vaccinated or unvaccinated mice immediately after virulent infection (Conge, Collin, Levy & Dubos, 1959). Equivalent numbers of organisms are also recovered from lungs of animals in both groups for many days after challenge. Moreover, a given infection produces identical numbers of pulmonary lesions in animals irrespective of whether they are vaccinated or not (Levy *et al.* 1961). Thus, in the case of mice, the resistance acquired by vaccination does not appear capable of greatly influencing the host's initial response to virulent challenge.

Recent studies in this laboratory show that this question may be approached more directly. Thus, animals may be consistently exposed to a respiratory infec-

* This investigation was supported in part by the United States–Japan Cooperative Medical Science Programme administered by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Department of Health, Education and Welfare.

tion composed of only a few virulent organisms. Under these conditions only a small number of untreated mice become infected. Thus, mice may be experimentally challenged under conditions analogous to those occurring in natural human infection. This technique makes it possible to test exactly whether the incidence of infection is significantly lowered by prior BCG vaccination.

The incidence of tuberculous infection was found to be identical in control and immunized mice. Hence, BCG vaccination did not appear to enhance the ability of mice to prevent the initial establishment of even a small number of virulent bacilli in their pulmonary tissue.

MATERIALS AND METHODS

The materials and methods used here have been described previously (Costello, Dubos & Schaedler, 1962; Costello, Hedgecock & Hamilton, 1962).

Animals

The NCS strain of Swiss mice was used in all phases of the study (Nelson & Collins, 1961). Female animals were obtained when 5–6 weeks old and used immediately. Animals were maintained on a commercial pellet diet (D & G pellets, Dietrich & Gambrill, Inc., Frederick, Md.) with unlimited access to tap water.

Vaccination

The Montreal Strain of BCG was obtained from the Institute of Hygiene, University of Montreal.* The vaccine was received in the lyophilized state and was used without subculture. Ampoules containing 118 mg. of freeze-dried bacilli were reconstituted with 50 ml. of albumin water. Samples of the reconstituted material were stored at $-60°$ C. in a technique similar to that described by Grover, Kim, Wiegshouse & Smith (1967). A tenfold dilution of the thawed vaccine was made before use and 0·2 ml. of the resulting suspension intraperitoneally injected into individual mice. Animals were vaccinated when received and 10 weeks later they, together with comparable untreated mice, were challenged with virulent mycobacteria.

Challenge

The H37Rv strain of *Mycobacterium tuberculosis* was originally obtained from The Trudeau Institute, Saranac Lake, New York. Subcultures were made in Kirchner's medium containing Tween 80 and bovine serum albumin. Before use, the growth from a 5-day culture was homogenized in a teflon-glass tissue grinder for 1 min. and the resulting suspension filtered through a 5 μ porosity Millipore filter. This filtered material was found to consist primarily of single bacilli suitable for aerosol administration. The number of living bacilli present in the infective material was determined at this time and again at the time of challenge infection. Material sufficient for all challenge infections was prepared at the same time. Samples were stored at $-60°$ C. until use. Immediately before use, a sample was removed from

* The authors are indebted to Dr Frappier of the Institute of Hygiene for making large quantities of vaccine available for this study.

storage, thawed and serial tenfold dilutions were made with albumin water in order to obtain the desired number of virulent organisms/ml.

Animals were infected in a Middlebrook type aerosolization chamber (Tri R Instrument Corporation, Rockville Centre, N.Y.) (Izumi, Costello & Dubos, 1969). The infection induced by nebulization of different quantities of organisms was determined in initial experiments which are detailed in the experimental section. Generally speaking, 10 ml. of infectious material was nebulized over a 60-min. period. The rate of infectious air flow was held constant at 20 l./min. Following nebulization of the infective inocula, the aerosol cloud was allowed to decay for 30 min. and the chamber decontaminated with UV light for a further 15-min. period.

For the experimental tests, vaccinated mice were marked and mixed with untreated mice before being placed in the infection basket of the aerosol chamber. Sixty mice were exposed to infection at one time. Following airborne exposure, the animals were removed, resegregated, and maintained in separate groups until killed.

Enumeration of organisms from lungs of mice

Unless otherwise indicated, mice were killed 4 weeks after infection. At this time the animal's lungs were aseptically removed and homogenized in 2 % bovine serum albumin water with the aid of a teflon-glass tissue grinder. Serial tenfold dilutions were made in albumin water. Equal volumes of each dilution were transferred to sterile screw cap tubes to which 2 ml. of base medium* containing 0·2 % agar was added. The tubes were incubated at 37° C. and the number of colonies which developed was determined after 3 weeks incubation.

Separation of the organisms of challenge from those of vaccination was accomplished by use of the differential inhibitor 2-thiophene-carboxilic acid hydrazide. The inhibitor, in water, was sterilized by filtration and added to the soft agar medium in a final concentration of 15 μg./ml.

Standardization of experimental conditions

Establishment of a reproducible infection was crucial to the experimental work described. The inoculum for nebulization was carefully standardized. The rate of nebulization and the flow of infectious air were kept constant. The same venturi apparatus was used for nebulization in all experiments. The animals used were of the same age and sex.

When these and like conditions were uniformly maintained, the dose of organisms required to induce pulmonary infection in some, but never all of the exposed animals was readily established. Moreover, these conditions could be reproduced at will.

* A base medium of the following composition was used in these experiments: $Na_2HPO_4 . 12H_2O$, 3·0 g.; KH_2PO_4 (Anh), 4·0 g.; sodium citrate.H_2O, 1·0 g.; ferric ammonium citrate (USP grade), 0·1 g.; $MgSO_4 . 7H_2O$, 0·1 g.; bacto asparagine, 2·0 g.; bacto tryptone, 5·0 g.; bacto agar, 2·0 g.; glycerin, 10·0 ml.; H_2O, to 1000 ml.

The following additions were made to each 100 ml. of melted medium prior to use: penicillin (10,000 unit/ml.), 0·1 ml.; lysozyme (0·1 % in 0·01 N-HCl), 1·0 ml.; bovine serum, 10·0 ml.

The results described were obtained in a number of different experiments. Since large groups of animals were used and since the results were remarkably consistent and reproducible, a high degree of confidence can be attached to them.

RESULTS

Exposure of mice to small doses of mycobacteria

The minimum quantity of mycobacteria required to initiate pulmonary tuberculous infection in mice was determined as follows.

In a typical experiment 6-week-old female mice were obtained and distributed into several large groups. Each group of animals was exposed to aerosol infection with a diminishing number of virulent human bacilli. To this end, serial tenfold dilutions of the suspension of virulent mycobacteria were made in albumin water. At successive times, individual groups of animals were exposed to the aerosol produced by nebulizing 10 ml. of each dilution. Thus, the first group of animals was exposed to the aerosol obtained from the most diluted suspension. After decontamination of the chamber these animals were removed and the second group of mice was exposed to aerosol infection with the next highest dilution and so forth. The last group of mice was exposed to the aerosol obtained by nebulization of the undiluted suspension. Five mice from each group were killed at various intervals following challenge and the numbers of organisms present in their lungs were determined. The results of this experiment are detailed in Tables 1 and 2.

Table 1. *Pulmonary infection with various aerosol exposures of virulent mycobacteria (H 37 Rv)*

Experiment	No. of viable bacilli in nebulized suspension (per ml.)	Exposure time* (min.)	Bacilli recovered from lungs†		Lungs containing bacilli positive/total
			Log.	S.E.	
1	$10^{7\cdot3}$	60	7·2	± 0·11	5/5
	$10^{6\cdot3}$	60	6·6	± 0·25	5/5
	$10^{5\cdot3}$	60	5·2	± 0·12	5/5
	$10^{4\cdot3}$	60	4·5	± 0·31	5/5
	$10^{3\cdot3}$	60	4·0	± 0·04	2/5
	$10^{2\cdot3}$	60	0·0	—	0/5
2	$10^{3\cdot3}$	45	5·0	± 0·06	2/5
	$10^{2\cdot3}$	45	0	—	0/5
	$10^{1\cdot3}$	45	0	—	0/5
	$10^{0\cdot3}$	45	0	—	0/5
3	$10^{5\cdot5}$	60	4·5	± 0·68	5/5
	$10^{5\cdot5}$	30	5·1	± 0·35	3/5
	$10^{4\cdot5}$	60	3·6	± 1·79	3/5
	$10^{4\cdot5}$	30	3·7	± 0·23	1/5

* The volume of suspension nebulized was approximately 9·9 ml./hr. for Expt. no. 1; 7·5 ml./45 min. for Expt. no. 2; and 9·4 ml./hr. for Expt. no. 3.

† Number of virulent mycobacteria (expressed as logs) recovered from lungs 4 weeks after challenge infection ± standard error of mean. Values are for 1 cc. of lung homogenate (out of a total 5 cc.). Only positive values (i.e. of lungs containing mycobacteria) are included in average.

In other experiments the incidence of infection in mice exposed to the aerosol mist for shorter intervals of time was determined. The same technical conditions were maintained in these experiments as in those just described. These results are also given in Tables 1 and 2.

Table 2. *Challenge organisms recovered from lungs of untreated mice following aerosol exposure to virulent mycobacteria*

Experiment	No. of viable bacilli in nebulized suspension (per ml.)	Exposure time (min.)	Time between challenge and sacrifice (wks.)	Mycobacteria recovered from lungs of individual animals* (log.)	Group average (log.)	No. of lungs containing mycobacteria positive/total
1	$10^{4\cdot3}$	60	1	1·3, 1·5, 1·5, 1·8, 1·9	1·6	5/5
			2	3·5, 3·5, 3·5, 3·7, —	3·5	4/4
			4	3·5, 4·0, 5·0, 5·0, 5·0	4·5	5/5
			10	5·9, 6·0, 6·3, 6·7, 7·0	6·3	5/5
			20	5·7, 7·0, 7·6, 8·0, 8·7	7·4	5/5
			40	7·0, 7·0, 9·3, 11·0, 11·0	9·0	5/5
	$10^{3\cdot3}$	60	1	0, 0, 0, 0·3, 0·5	0·4	2/5
			2	0, 2·5, 2·8, 3·3, 3·3	3·0	4·5
			4	0, 0, 0, 3·9, 4·0	4·0	2/5
			10	0, 0, 0, 4·0, 5·7	4·8	2/5
			20	0, 5·6, 5·9, 6·9, 7·5	6·5	4/5
			40	0, 0, 5·9, 6·3, 8·5	6·9	3/5
	$10^{2\cdot3}$	60	1–10	0, 0, 0, 0, 0	—	All 0/5
2	$10^{3\cdot3}$	45	1	0, 0, 0, 0·1, 0·3	0·2	2/5
			2	0, 0, 0, 0, 0	—	0/5
			4	0, 0, 0, 4·5, 5·6	5·0	2/5
			10	0, 0, 0, 0, 6·0	6·0	1/5
	$10^{2\cdot3}$	45	1–10	0, 0, 0, 0, 0	—	All 0/5

* Number of virulent mycobacteria (expressed as logs) recovered from individual lungs after challenge infection. See Table 1 for explanation.

The first experiment described in Table 1 illustrates the type and severity of infection which occurred in animals following exposure to aerosols of different bacterial densities. The results presented in this table indicate the proportion of animals which contracted infection and the number of organisms recovered from their lungs 4 weeks after challenge.

Infection occurred in all animals exposed for 60 min. to aerosols produced by nebulizing suspensions containing from $10^{7\cdot3}$ to $10^{4\cdot3}$ bacilli/ml. The number of bacilli recovered from the lungs was roughly proportional to the size of the infective inoculum. In contrast, only two out of five animals exposed to $10^{3\cdot3}$ bacilli/ml. showed organisms in their lungs and none of those exposed to the next higher dilution were infected. The minimal infective dose, established in this manner, is defined as the number of bacilli which, when nebulized under standardized conditions, causes infection in some but not all of the animals exposed to it.

The second experiment described in Table 1 presents the results of a similar although independent experiment. Dilutions of the same frozen stock of challenge

organisms were used. Animals were exposed to the aerosol for 45 min. instead of 1 hr. Again in this experiment, some of the animals exposed to the aerosolization of $10^{3\cdot 3}$ bacilli/ml. became infected while no animals were infected when exposed to $10^{2\cdot 3}$ or fewer mycobacteria.

Fewer animals exposed to this shorter length of challenge were infected; this may be seen better in Table 2, where the results of the two experiments are described in greater detail. It is worth noting, however, that the number of organisms present in the lungs of infected animals in experiment two was as high, or indeed, slightly higher, than in infected mice in the first experiment.

The results detailed for experiment three in Table 1 further illustrate that the infectivity of small doses of mycobacteria administered by the respiratory route depends not only on the number of infective particles in the aerosol cloud but also on the length of time that the animals are exposed to it. Thus, as is shown by the results of this experiment, aerosols of suspensions which infected all mice when nebulized for 1 hr. were able to infect only some animals when the length of exposure was decreased to 30 min. Similarly, a serial dilution of this suspension infected some animals when it was administered for 1 hr. but was only sporadically infective when the length of exposure was shortened.

The results given in Table 2 illustrate these points in greater detail. In this table the sequential course of infection is followed in animals exposed to moderate and minimal respiratory tubercular challenge. For illustrative purposes, the actual number of bacilli (in logs) recovered from each animal's lung at various times after challenge is given.

It may be observed that all animals exposed to aerosols obtained by nebulization of a moderate number of bacilli ($10^{4\cdot 3}$ bacilli/ml.) were infected. The pulmonary infection progressed in a uniform manner in these mice with increasingly large numbers of virulent organisms being recovered from the animal's lungs at every test period.

In contrast, only some 60 % of the mice exposed to the smaller challenge dose became infected. The proportion of infected to non-infected animals present was found to be similar at all intervals of time after infection. This finding, i.e. that the relative number of animals whose lungs were infected did not increase with time, is of especial importance, since it suggests that retarded or delayed pulmonary infection did not occur in any significant number of animals.

The growth of organisms in the lungs of these infected mice progressed at approximately the same rate as it did in the tissues of mice infected by heavier aerosol exposure. Thus, only small numbers of organisms were present in infected lungs the first week after challenge. Four weeks later, extensive multiplication of the virulent mycobacteria had occurred in these lungs and the infected animals were readily distinguishable from their non-infected cage mates.

Incidence of infection in BCG vaccinated mice

The effect of BCG immunization on infectious morbidity was studied in mice vaccinated by peritoneal administration of living BCG.

The *in vivo* growth of the vaccine was determined in preliminary experiments.

Six-week-old female NCS mice were obtained and different groups of animals were injected with one of several dilutions of vaccine. At intervals thereafter, five animals of each group were killed and the number of BCG bacilli contained in their organs determined.

Both spleens and lungs of animals injected with heavier doses of vaccine were immediately infected and large numbers of bacilli were subsequently recovered from either organ. In all experiments described animals were injected with $10^{5 \cdot 7}$ BCG bacilli/mouse. Ten weeks after injection of this dose of vaccine, the lungs of immunized animals still retained culturable vaccine bacilli, although in low number, and their spleens contained an average of $10^{3 \cdot 8}$ bacilli. These animals were found to be highly immune as measured by resistance to the pulmonary growth of virulent mycobacteria given by the respiratory route, and to intravenous injection of massive quantities of human bacilli.

Large numbers of mice were used in the main vaccination experiments. For this purpose, 6-week-old mice were obtained and divided into two groups. One group of animals received 0·2 ml. of BCG vaccine by peritoneal injection; the other group was left untreated. Exactly 10 weeks after immunization, animals of both groups were exposed to aerosol challenge infection. For this purpose vaccinated and non-vaccinated mice were mixed, placed in the aerosol chamber and exposed to the respiratory challenge infection previously found (see above) to infect some but not all of the untreated animals. Animals were killed 4 weeks after challenge infection and the number of virulent bacilli present in their lungs determined. This same procedure was repeated at weekly intervals. The results of several typical experiments are presented in Tables 3 and 4.

The actual numbers of mycobacteria present in samples of lung tissue homogenate in a representative experiment are given in Table 3. The experimental groups in this case consisted of ten untreated and ten vaccinated mice. Animals were killed 4 weeks after challenge infection. Their lungs were removed, homogenized and the numbers of virulent organisms present in the homogenate determined.

As may be seen, one-half of the untreated animals were infected during the challenge exposure. Large numbers of bacilli were recovered from all dilutions of homogenate of these infected lungs. No organisms were recovered from the lungs of the remaining five animals.

Similarly, four out of the ten vaccinated animals acquired infection from exposure to the challenge aerosol. Organisms were found only in the lower dilutions of lung homogenate of these mice. Thus, significantly fewer organisms were present in the infected lungs of vaccinated mice than were recovered from lungs of non-immunized control animals.

The effect of prior BCG vaccination on the incidence of pulmonary infection by small numbers of virulent bacilli is further detailed in Table 4 where the results of three successive experiments are illustrated.

It may be seen that the same relative number of vaccinated or unvaccinated animals were infected in each experiment. The rate of infection of untreated animals varied in the three experiments from a high of 95 % to a low of 38 %,

probably due to slight, although indeterminate, variations in technical conditions.

It is apparent that the resistance acquired by vaccination with BCG had little significant effect on the animal's ability to prevent establishment of the organisms of challenge within their pulmonary tissue. However, vaccination retarded the progress of the pulmonary infection even though it did not prevent its initial establishment.

Table 3. *Organisms present in lungs of normal and BCG vaccinated animals after minimal infective challenge*

Organisms recovered from lungs of individual mice*

Mouse no.	Prior treatment	Dilutions of lung homogenate: Un	−1	−2	−3	No. of bacilli recovered (log.)
		No. of bacilli				
1	Non-vaccinated	TN‡	C‡	TN	50+	> 4·0
2		0	0	0	0	—
3		C	TN	TN	TN	> 4·0
4		0	0	0	0	—
5		0	0	0	0	—
6		TN	TN	TN	TN	> 4·0
7		0	0	0	0	—
8		TN	TN	TN	50+	> 4·0
9		TN	TN	TN	TN	> 4·0
10		0	0	0	0	—
1	BCG vaccinated†	0	0	0	0	—
2		0	0	0	0	—
3		0	0	0	0	—
4		TN	50+	4	0	2·6
5		0	0	0	0	—
6		TN	TN	50	5	3·7
7		TN	TN	28	0	3·5
8		0	0	0	0	—
9		0	0	0	0	—
10		TN	TN	11	0	3·0

* Number of virulent mycobacteria recovered from individual mice 4 weeks after challenge. Challenge infection was produced by nebulization of 10 ml. suspension containing $10^{3\cdot3}$ bacilli/ml. over a 60-min. period. Values refer to actual number of bacilli present in 1 cc. of lung homogenate (out of a total of 5 cc.) or of 1 cc. of each serial dilution of this suspension.

† Animals were peritoneally injected with 0·2 ml. of Montreal strain BCG 10 weeks prior to challenge. Individual animals received 0·047 mg. of vaccine containing $10^{5\cdot7}$ cultivable bacilli.

‡ TN indicates value too numerous to count; C indicates contaminated sample.

It is of interest to compare the relative retardation of bacterial growth in lungs of vaccinated animals infected with a minimum number of virulent bacilli with those of animals infected by aerosol exposure to infective clouds containing approximately 100 times as many virulent bacilli. Experiments, not to be detailed here, have shown that 4 weeks after challenge infection the lungs of heavily infected vaccinated animals contain *ca.* 1·2–1·6 log. fewer organisms than lungs of

unvaccinated mice. In a typical experiment, for example, animals were infected with an aerosol produced by nebulizing $10^{5\cdot5}$ virulent bacilli. Lungs of unvaccinated mice were found to contain an average of $10^{6\cdot0}$ virulent bacilli when tested 4 weeks after challenge, whereas lungs of BCG vaccinated mice infected at the same time contained an average of $10^{4\cdot6}$ virulent organisms. This relative difference is essentially the same as that found in the experiments described in Table 4.

Table 4. *Incidence of infection of normal or BCG vaccinated mice when exposed to small numbers of virulent mycobacteria*

Experiment	Vaccination*	No. of viable bacilli in nebulized suspension	No. of animals challenged	Animals with infected lungs		Organisms recovered from infected lungs†,‡	
				No.	Per cent	Log.	S.E.
1	—	$10^{3\cdot3}$	20	19	95	3·9	± 0·23
	BCG	$10^{3\cdot3}$	17	16	94	3·1	± 0·19
2	—	$10^{3\cdot3}$	20	15	75	4·1	± 0·19
	BCG	$10^{3\cdot3}$	21	18	85	3·1	± 0·10
3	—	$10^{3\cdot3}$	13	5	38	4·0	± 0·39
	BCG	$10^{3\cdot3}$	20	8	40	3·0	± 0·20

* Animals were peritoneally injected with 0·2 ml. of Montreal Strain BCG 10 weeks prior to challenge. Individual animals received 0·047 mg. of vaccine containing $10^{5\cdot7}$ cultivable bacilli.

† Number of virulent mycobacteria (expressed as logs) recovered from lung 4 weeks after challenge infection ± standard error of the mean. Values are for 1 cc. of lung homogenate (out of a total of 5 cc.).

‡ P values comparing vaccinated to control groups for the three experiments are: Expt. no. 1, $< 0\cdot02$; Expt. no. 2, $< 0\cdot01$; Expt. no. 3, $< 0\cdot05$.

DISCUSSION

The results described in this paper give direct evidence that morbidity to respiratory tuberculosis is not significantly lowered in mice by vaccination with living BCG.

Other investigators have shown that the early pulmonary infection which occurs in immune and non-immune animals is quantitatively similar. Thus, it has been previously postulated that the initial establishment of tubercle bacilli within the lungs of mice would not be greatly influenced by the hosts' immune response (Levy *et al.* 1961). The results of the experiments described in this paper confirm these deductions. It should be pointed out that, in the study just quoted, relatively large infective inocula were used. Thus, any small protective capacities of the immunized animal were likely to have been overwhelmed by the organisms of challenge. That this consideration is without merit, however, is apparent from the results described here since in these experiments immunized pulmonary tissue was unable to prevent the establishment of the few, perhaps even single, infectious particles that were administered.

Although the primary lodgement of virulent bacilli in pulmonary tissue was not

prevented by prior vaccination with BCG, the subsequent growth of the challenge organisms was sharply limited. The fact that significantly fewer challenge organisms were present in infected lungs of vaccinated animals at the time of testing is evidence that these mice had acquired a high degree of immunity.

The conditions of vaccination used in these experiments deserves comment. Larson & Wicht (1962) found that the resistance attained by animals was materially affected by the route by which the vaccine was administered; this was not supported by our findings (Izumi *et al.* 1969). In this regard it should be pointed out that large quantities of BCG were administered in our experiments and, irrespective of the site of inoculation, large numbers of vaccine organisms were recovered from the spleen and lungs at the time of challenge. None the less, it is possible that mice vaccinated under other conditions may respond differently and that their pulmonary tissue would be able to inhibit, either partly or completely, the establishment of small infective inocula.

It should be emphasized, moreover, that our conclusions are necessarily valid only for Swiss strain mice and may have limited bearing on the responses of other experimental animals to tuberculous infection. Indeed, it is not unlikely that other species of animals may respond in an entirely different fashion. In this regard, it should be noted that epidemiological studies suggest that the primary incidence of tuberculous disease in humans may be greatly lowered by vaccination with BCG.

Finally, the small quantity of virulent bacilli required to induce infection in untreated mice merits brief comment. Albino mice are presumed to have a high innate resistance to infection with tubercle bacilli. This belief is largely based on the finding that massive quantities of virulent bacilli must be administered to these animals to cause mortality. There is reason to believe, however, that the actual resistance of mice to tuberculous infection is greatly overestimated. Thus, it has been found that splenic infection can be uniformly established in mice injected intraperitoneally with as few as 6 ± 3 culturable bacilli (R. Costello & S. C. Slats, data to be published). Furthermore, lungs of mice can be consistently infected following intranasal instillation of suspensions of mycobacteria so dilute that organisms are recovered only occasionally on their culture (Gray & Mattinson, 1952). The results described in this present paper also suggest that lungs of mice are highly susceptible to respiratory infection. Thus, the aerosol obtained from nebulizing very dilute suspensions of virulent human bacilli can induce infection in at least some mice.

In this regard it is probably not possible to determine the exact number of bacilli inhaled by each animal during exposure to the small infective aerosol. From the number of bacilli present in the nebulized inocula, and the average nominal recovery values (Rosebury, 1947) as ascertained in separate experiments, however, it can be roughly estimated that animals exposed to the minimal infective aerosol (the quantity which caused infection of some but not all of the challenged mice) contacted between 0·2 and 2 virulent organisms. With an infective dose of this small magnitude it is likely that some animals inhaled single infective particles whereas others were exposed to none.

The results presented yield no direct evidence concerning the factors involved in vaccinal resistance to tuberculosis. It should not be presumed, however, from the failure of vaccinated animals either to prevent the establishment of tuberculous infection, or to prevent the pulmonary growth of virulent organisms, that the immune state which develops in mice is necessarily insignificant. It has been repeatedly demonstrated, for example, that BCG vaccination confers on mice a significant ability to limit the intraorgan spread and extrapulmonary growth of virulent bacilli. Furthermore, the average survival time of animals infected with virulent bacilli is greatly prolonged by prior BCG vaccination. In mice, as in men, the most important effect of vaccination may be to retard the solemn progression of disease.

The authors are indebted to their colleague, Dr Takehiko Sakurami, for theoretical and technical assistance with the aerosol infection apparatus. This work was performed in the laboratories of Dr Rene Dubos.

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J. Hyg., Camb. (1970), **68**, 601
Printed in Great Britain

Salmonella isolations in abattoirs in Greece

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(*Received* 4 *June* 1970)

SUMMARY

The prevalence of salmonellas in drain swabs from three abattoirs of Athens was studied with the use of conventional methods of *Salmonella* isolation as well as with a new procedure which involves secondary enrichment from the usual selenite broth or Muller–Kauffmann's tetrathionate broth in Rappaport's medium slightly modified.

In all groups studied the secondary enrichment in Rappaport's medium led to an increase in the number of positive swabs, in the number of *Salmonella* serotypes, and in the total number of strains isolated.

The frequency of *Salmonella* isolations was higher in samples from abattoirs killing only pigs and lower in samples from abattoirs killing only cattle or only sheep.

The predominant serotype in abattoirs dealing with cattle was *Salmonella tennessee*, and *S. typhimurium* in abattoirs dealing with sheep. No predominant serotype was found in samples from abattoirs dealing mostly with pigs.

S. abony, *S. drypool*, *S. emek*, *S. indiana*, *S. muenchen* and *S. tennessee* were isolated for the first time in Greece.

INTRODUCTION

The prevalence of salmonellas in abattoirs has been studied by several investigators. In these studies different enrichment and selective media were used but the incubation temperature was usually 37° C. (Jones, Bennet & Ellis, 1961; Harvey & Phillips, 1961; Valette, 1961; Report, 1964; Papadakis, 1965; Papadakis, 1968; and others). Harvey & Thompson (1953), however, who used selenite F broth, reported a better yield of salmonellas, especially from river water and sewage, when the enrichment medium was incubated at 43° C. Since then, this incubation temperature has been used with profit by some authors (Georgala & Boothroyd, 1964; Harvey & Price, 1968; Harvey, Price, Foster & Griffiths, 1969). The advantage of incubating the enrichment media at 43° C. was also evident in a recent comparative study of *Salmonella* isolations from faeces and minced meat in eight European laboratories (Edel & Kampelmacher, 1968).

From a different point of view, several investigators have reported that a secondary enrichment in selenite broth or tetrathionate broth also tends to increase the yield of salmonellas from food or highly contaminated material (Jameson, 1962, 1963; Edel & Kampelmacher, 1968; Martin & Ewing, 1967; Harvey & Price,

1967; and others). In this paper, we report our findings from a comparative evaluation of the isolation of salmonellas from abattoir drains by three different procedures: first, by the use of conventional enrichment media incubated at 37° C.; secondly, by conventional enrichment media incubated at 43° C.; and thirdly, after secondary enrichment in Rappaport's medium. This medium, while allowing a satisfactory growth of salmonellas, is strongly inhibitory to the usual competing organisms (Rappaport, Konforti & Navon, 1956; Vassiliadis, 1968). The frequency of isolation of the various salmonella strains and serotypes in abattoirs in Greece according to species of slaughtered animals is also reported.

MATERIAL AND METHODS

Laboratory technique

The following media have been used: mannitol–selenite F broth (Oxoid), Muller–Kauffmann's tetrathionate broth (MK) containing 1/100,000 of brilliant green and Rappaport's medium (Rappaport *et al.* 1956). In the preparation of the last, only 2·5 ml. of 'solution C' instead of 3 ml. were added to 100 ml. of 'solution A'. We introduced this modification because we have noticed that this amount of malachite green, while still inhibitory to competing organisms, allows a better growth of salmonellas. Enrichment cultures in selenite broth were incubated at 37° C. for 2 days; those in MK broth were incubated at 37° C. and, in a great number of samples, at 43° C. as well, also for 2 days. All secondary enrichments were made in Rappaport's medium, by transferring into it an inoculum, with a 3–4 mm. loop, from the primary enrichment media. Incubation of all the secondary enrichments was made at 37° C. for 1 day. The enrichment and secondary enrichment media were subcultured either on Wilson and Blair's bismuth sulphite agar modification (Mackie & McCartney, 1956) (for 74 drain swabs of groups B and C—see Tables 2 and 3) or on Hynes's deoxycholate-citrate agar (Hynes, 1942) (for 20 drain swabs of group A—see Table 1).

Drain swabs from abattoirs

Abattoir drains were examined by Moore's gauze-swab technique (Moore, 1948). Swabs were placed for a period of 2–4 days in drains of three abattoirs in Athens. In one of these abattoirs there were three different compartments in which cattle, sheep (and very few goats) and pigs were separately killed. Independent drains, however, existed for only the first two compartments, while the drain of the pig compartment received the draining of the second one. In another abattoir, pigs were almost exclusively slaughtered. In a third abattoir cattle and sheep (and very few goats) were killed in one compartment with an independent drain and pigs in another compartment whose drain received the draining of the former. A total of 94 abattoir drain swabs were examined and distributed according to their origin as follows: 14 swabs from abattoir compartments killing only pigs; 38 from compartments killing only cattle; 34 from compartments killing only sheep (and a few goats); and the remaining eight swabs from abattoir compartments in which more than one animal species was slaughtered. No disinfectant was used in any of these abattoirs during the washing.

The drain swabs were divided, in the laboratory, into two equal parts, each of which was introduced into a screw-capped jar containing the enrichment medium.

Our examinations were carried out between March 1969 and February 1970, but they were interrupted for about $2\frac{1}{2}$ months during the summer. The approximate number of animals slaughtered during the days the swabs were left in position was as follows: 1900 pigs, 3800 cattle, 30,000 sheep, and 300 goats. All the cattle, pigs and goats were raised in various parts of Greece, while the great majority of the slaughtered sheep were imported from Hungary.

RESULTS

Isolations in relation to the procedure employed

The 94 abattoir drain swabs were classified into three groups, A, B and C.

Group A drain swabs

In this group, 20 drain swabs were examined by enrichment in selenite broth and in tetrathionate broth incubated at 37° C., followed by secondary enrichment in the modified Rappaport's medium. During the 2-month period that the swabs of group A were left in position in the abattoirs, 350 cattle, 4000 sheep (and goats)

Table 1. Salmonella *isolations from 'group A' abattoir drain swabs*

(Among the 20 large swabs of 'group A' 11 were negative in all procedures.)

No. of isolated strains by serotype	Enrichment medium*			
	S 37° C.	MK 37° C.	S 37° C./R	MK 37° C./R
S. abony	0	0	1	1
S. braenderup	0	0	0	1
S. indiana	0	0	1	1
S. senftenberg	0	0	0	1
S. tennessee	0	0	1	2
S. typhimurium	0	0	1	5
Total no. of isolated strains	0	0	4	11
Total no. of serotypes	0	0	4	6
Total no. of positive swabs	0	0	4	9
Swabs positive as percentage of total	0	0	20·0	45·0

* S 37° C. or MK 37° C. = enrichment in selenite broth or Muller–Kauffmann's tetrathionate broth, incubated at 37° C. for 48 hr.; S 37° C./R or MK 37° C./R = secondary enrichment in Rappaport's broth from selenite broth or MK broth respectively. The growths from the enrichment and secondary enrichment media were subcultured on Hynes's modification of deoxycholate citrate agar.

and 300 pigs were killed. The results of the isolations in this group are shown in Table 1. The secondary enrichment in Rappaport's medium led to a significant number of salmonella isolations particularly when combined with primary enrichment in MK tetrathionate broth. It should be noted that no salmonellas were isolated when only selenite or tetrathionate broth was used as enrichment medium.

This complete failure may be attributed to the use, in this particular group of examinations (group A), of large swabs which, when added to the enrichment media, covered more than 45 % of the total volume. As a result, an abundance of competing bacteria were present in the subcultures on the Hynes's agar, while these germs were inhibited to a great extent by the secondary enrichment in Rappaport's broth.

Two different serotypes from a single swab were isolated twice, while in seven other instances, only one serotype was isolated from every positive swab.

Table 2. Salmonella *isolations from 'group B' abattoir drain swabs*

(Among the 21 regular swabs of 'group B' 11 were negative in all procedures.)

No. of isolated strains by serotype	Enrichment medium*			
	S 37° C.	MK 37° C.	S 37° C./R	MK 37° C./R
S. braenderup	0	0	2	1
S. derby	2	0	0	0
S. senftenberg	1	0	1	0
S. tennessee	0	3	1	4
S. typhimurium	0	1	2	2
Total no. of isolated strains	3	4	6	7
Total no. of serotypes	2	2	4	3
Total no. of positive swabs	3	4	6	7
Swabs positive as percentage of total	14·3	19·0	28·6	33·3

* See footnote on Table 1.

The growths from the enrichment and secondary enrichment media were subcultured on Wilson and Blair's bismuth sulphite agar.

Group B and group C drain swabs

In these groups the volume of the swab added to the enrichment media did not exceed 20 % of the total volume and all subcultures from enrichment and secondary enrichment media were made on bismuth sulphite agar. During the 2-month period that the swabs of group B were left in position in the abattoirs, the approximate number of killed animals was 700 cattle, 11,400 sheep (and goats) and 300 pigs while, during the 4½ month period that the swabs of group C were left in position, the number of slaughtered animals was approximately 1300 pigs, 2750 cattle and 14,900 sheep (and goats). The results of the examinations are summarized in Tables 2–4. It can be seen from these tables that secondary enrichment in Rappaport's medium increases the frequency of *Salmonella* isolations from abattoir drain swabs to a considerable extent. In addition, our results show the superiority of 43° C. over 37° C. as the incubation temperature for enrichment in Muller–Kauffmann's tetrathionate broth.

Among the 10 positive swabs of group B, seven yielded one serotype each and the remaining three two serotypes each. Among the 47 positive swabs of group C, 27 yielded one serotype, 16 two serotypes, 3 three serotypes and the last one four serotypes.

Salmonella isolations in relation to the species of slaughtered animal

The frequency distribution of the serotypes and strains of salmonellas isolated from all drain swabs in relation to the species of killed animals is shown in Table 5. The frequency of positive swabs was higher in abattoir compartments killing only pigs and lower in abattoir compartments in which only cattle or sheep were killed.

Table 3. Salmonella *isolations from 'group C' abattoir drain swabs*

(Among the 53 regular swabs of 'Group C' 6 were negative in all procedures.)

No. of isolated strains by serotype	Enrichment medium*			
	MK 37° C.	MK 43° C.	MK 37° C./R	MK 43° C./R
S. abony	7	7	7	7
S. braenderup	3	2	1	0
S. bredeney	0	2	1	1
S. derby	2	1	5	2
S. drypool	2	2	1	1
S. emek	2	2	6	8
S. infantis	1	2	3	3
S. kottbus	1	1	1	2
S. meleagridis	0	0	1	0
S. muenchen	2	0	3	2
S. oranienburg	0	2	0	1
S. richmond	0	1	1	1
S. senftenberg	1	1	2	3
S. tennessee	8	10	7	9
S. typhimurium	4	4	7	7
S. westerstede	0	1	0	0
*S.*O-6, 7: unidentified	0	1	0	0
*S.*O-8: unidentified	0	0	1	1
Total no. of isolated strains	33	39	47	48
Total no. of serotypes	11	15	15	14
Total no. of positive swabs	32	39	43	45
Swabs positive as percentage of total	60·4	73·6	81·1	84·9

* MK 37° C. or MK 43° C. = enrichment in Muller–Kauffmann's tetrathionate broth incubated at 37° C. or 43° C. respectively; MK 37° C./R or MK 43° C./R = secondary enrichment in Rappaport's broth from MK enrichment incubated at 37 °C. or 43° C. respectively.

The growths from the enrichment and secondary enrichment media were subcultured on Wilson and Blair's bismuth sulphite agar.

It may also be added that *Salmonella* isolations were in general more frequent in group C than in group B. Although an explanation of this fact may be found in the correspondingly larger number of pigs and cattle killed, further exploration of the effect of the number of slaughtered animals by individual species on the frequency of salmonella isolations has not been possible because of the diversity of the conditions and the different laboratory techniques.

Table 4. *Comparative evaluation of four procedures for* Salmonella *isolation from abattoir drain swabs*

I. MK 43° C. versus MK 37° C.	
Total number of swabs (group C)	53
Swabs positive on MK 43° C.	39
Swabs positive on MK 37° C.	32
Swabs positive on MK 43° C. and negative on MK 37° C.	9
Swabs positive on MK 37° C. and negative on MK 43° C.	2
χ^2 for paired samples with Yates correction	3·27 $P < 0{\cdot}10$
II. MK 37° C./R versus MK 37° C.	
Total number of swabs (groups B and C)	74
Swabs positive on MK 37° C./R	50
Swabs positive on MK 37° C.	36
Swabs positive on MK 37° C./R and negative on MK 37° C.	14
Swabs positive on MK 37° C. and negative on MK 37° C./R	0
χ^2 for paired samples with Yates correction	12·07 $P < 0{\cdot}001$
III. MK 43° C./R versus MK 43° C.	
Total number of swabs (group C)	53
Swabs positive on MK 43° C./R	45
Swabs positive on MK 43° C.	39
Swabs positive on MK 43° C./R and negative on MK 43° C.	6
Swabs positive on MK 43° C. and negative on MK 43 °C./R	0
χ^2 for paired samples with Yates correction	4·17 $P < 0{\cdot}05$

Relative efficiency of the four compared procedures for *Salmonella* isolation from abattoir drain swabs group C, assuming efficiency of MK 37° C. = 100.
MK 37° C. = 100. MK 43° C. = 122. MK 37° C./R = 134 MK 43° C./R = 141.

DISCUSSION

It can be seen from Tables 1 to 4 that a secondary enrichment in Rappaport's medium, from either selenite broth incubated at 37° C. or Muller–Kauffmann's tetrathionate broth incubated at 37° C. or 43° C., led to a significant increase in the number of positive swabs as well as in the number of *Salmonella* strains isolated. The advantages of the secondary enrichment in Rappaport's broth were not substantially affected by the conditions and media of the primary enrichment. It may also be noted that the superiority of the procedures involving secondary enrichment in Rappaport's medium was apparent in all three groups of abattoir drain swabs (groups A, B and C), although the frequency of isolations was generally lowest in group A, presumably because of the excessive size of the swabs, and highest in group C, possibly because of the correspondingly larger number of pigs and cattle. The secondary enrichment in Rappaport's medium does not seem to alter the pattern of serotypes isolated. Although the data in Table 2 suggest that secondary enrichment favours the isolation of *S. braenderup* and does not favour the isolation of *S. derby*, the data in Table 3 point in the opposite direction and indicate the importance of chance factors.

As mentioned in the Introduction, other investigators have already shown that secondary enrichment in selenite broth or tetrathionate broth increases the relative efficiency of salmonella isolations. Although there is no strict comparability between the results of the various investigations and our examinations were so far

restricted to abattoir drain swabs, our results suggest that secondary enrichment in the slightly modified Rappaport medium may improve the efficiency of *Salmonella* isolations at least as much as secondary enrichment in other more conventional media.

Table 5. Salmonella *isolations from abattoir drain swabs according to the species of the slaughtered animals*

No. of isolated strains by serotype	Abattoirs killing only pigs	Abattoirs killing only cattle	Abattoirs killing sheep and goats	Abattoirs killing various species	All abattoirs
S. abony	1	3	4	0	8
S. braenderup	3	1	0	2	6
S. bredeney	0	0	1	1	2
S. derby	3	1	1	3	8
S. drypool	2	1	0	1	4
S. emek	2	2	1	3	8
S. indiana	1	0	0	0	1
S. infantis	1	0	0	2	3
S. kottbus	0	2	0	0	2
S. meleagridis	0	0	1	0	1
S. muenchen	1	0	0	3	4
S. oranienburg	1	0	0	1	2
S. richmond	0	0	1	0	1
S. senftenberg	3	2	0	1	6
S. tennessee	1	17	2	1	21
S. typhimurium	1	5	10	0	16
S. westerstede	1	0	0	0	1
*S.*O-6, 7: unidentified	0	0	0	1	1
*S.*O-8: unidentified	0	1	0	0	1
Total no. of isolated strains	21	35	21	19	96
Total no. of serotypes	13	10	8	11	19
Total no. of positive swabs	14	24	21	7	66
Total no. of swabs	14	38	34	8	94
Swabs positive as percentage of total	100·0	63·2	61·8	87·5	70·2
Isolated strains per positive swab	1·5	1·5	1·0	2·7	1·5

The favourable influence of incubation at 43° C. rather than at 37° C. upon the primary enrichment medium has been stressed in other publications already mentioned. The results of our series of examinations in group C of abattoir drain swabs confirm this superiority and suggest that this fact holds equally well for samples from abattoir drains enriched in MK medium.

The frequency of *Salmonella* isolations was highest in abattoir compartments in which almost exclusively pigs were killed, as compared to abattoir compartments in which only cattle or only sheep and goats were killed (Table 5). The proportion of positive swabs from abattoirs killing only pigs (14/14 or 100 %) differed significantly from the corresponding proportion in abattoirs killing only cattle (24/38 or 63·2 %; $\chi^2 = 5{\cdot}30$ $P < 0{\cdot}05$) and from that in abattoirs killing only sheep and

goats (21/34 or 61·8%; $\chi^2 = 5{\cdot}54$ $P < 0{\cdot}05$). The importance of the findings is emphasized by the fact that the total number of slaughtered pigs was considerably smaller (1900) than the corresponding number for the cattle (3800) and the sheep and goats (30,300).

In the Report of the British Working Party of the Public Health Laboratory Service (Report, 1964), a different relation was found between the frequency of salmonella isolations and the species of animals killed. Thus, it was observed that salmonellas were isolated more often from abattoirs killing a large proportion of cattle and less often from abattoirs in which more sheep were killed, while the influence of the proportion of pigs killed proved more difficult to assess. This difference may be accounted for by the results of earlier studies in Greece which show that a high proportion of pigs, but a very low proportion of cattle, sheep and goats, were contaminated with salmonellas (Patéraki, Politi & Vassiliadis, 1966; Vassiliadis, Patéraki & Politi, 1969). It should be noted, however, that although the proportion of positive swabs was practically the same in abattoirs killing either only cattle or only sheep and goats, the number of strains isolated was considerably higher amongst the former (strains isolated per positive abattoir drain swab: cattle = 1·5; sheep = 1·0).

The highest number of different serotypes (13) was found among the 21 strains isolated from abattoir compartments killing only pigs. No predominance of any single serotype was noted in this group. By contrast, the serotype most frequently isolated (10 times) from abattoirs dealing with sheep was *S. typhimurium*, and *S. tennessee* the most frequently isolated (17 times) from those dealing with cattle.

The following serotypes were isolated for the first time in Greece: *S. abony*, *S. drypool*, *S. emek*, *S. indiana*, *S. muenchen* and *S. tennessee*.

We wish to express our thanks to Dr L. LeMinor of the Pasteur Institute, Paris, Director of the International Salmonella Reference Centre, for his kindness in confirming our identification of some of the reported serotypes which were isolated for the first time in Greece.

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Printed in Great Britain

Sewer and drain swabbing as a means of investigating salmonellosis

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(*Received* 12 *June* 1970)

SUMMARY

The use of gauze swabs in drains or sewers to clarify the path followed by a salmonella from source to human host has been reviewed in the light of experience gained in Cardiff over 15 years. This period has seen a marked change in attitudes to salmonella epidemiology in that infected food is now regarded as of greater importance than infected food handlers. In these 15 years, butchers, abattoirs and knackers' yards, markets and bakehouses have been monitored. In the bakehouse survey the existence of staff infection was demonstrated by sewage examination. Sewage investigation has also been used to show frequent entry of salmonellas into households in a residential estate. The estate was carefully chosen to exclude salmonellas from industrial sources and shops. The frequent finding of infection in this sewage implies that a commonly consumed heavily infected food item is involved. The wide range of serotypes isolated suggests an animal usually fed on infected animal feed. Poultry and pigs are put forward as animals liable to spread salmonellosis to man.

It is hoped that clarification of the salmonella pathway may eventually lead to measures likely to prevent the transmission of infection to man. It is also suggested that swabs placed in abattoir drains serve as an economical method of obtaining warning of a persistent build-up of contamination. The persistence of a serotype in an abattoir is not infrequently followed by human infection.

INTRODUCTION

The use of gauze swabs (Moore, 1948) for investigating salmonellosis in food premises was suggested by Moore, Perry & Chard (1952). In Cardiff, in 1955, such swabs placed in drains were employed to show the presence of *Salmonella typhimurium* in the environment of a butcher's shop suspected of selling infected meat. This incident led us to initiate a swab survey of bakehouses in 1955–7. The investigation demonstrated the entry of salmonellas in raw ingredients of confectionery manufacture and the existence of minor or latent infections in members of bakery staff (Harvey, 1957; Harvey & Phillips, 1961). The bakery survey led to a similar study of Glamorgan abattoirs in 1957–9. In this investigation, phage-typing of strains of *S. typhimurium* enabled a detailed comparison to be made between abattoir and human isolations. The timing of recovery of the same phage-

types from abattoir and man strongly suggested a causal association (Harvey & Phillips, 1961). Gauze swabs also proved of value in demonstrating the presence of *S. typhimurium*, type 12, in the environment of 15 out of 54 shops during an outbreak of salmonella food poisoning in Cardiff in 1960. Human infections in the city tended to be clustered round shops from which *S. typhimurium*, type 12, was isolated (Harvey, Price, Bate & Allen, 1963).

It is convenient to consider salmonella epidemiology in terms of a cycle. The importance of the various parts of the cycle are open to conjecture and argument. If we suggest, however, that one of the pathways travelled is:

Animal food → Animals on farms,
→ Animals at abattoirs,
→ Meat wholesalers and retailers,
→ Man,
→ Sewage,
→ Sewage polluted water,

a number of situations emerge which are worth monitoring by the swab technique.

The salmonella content of animal feed requires continuous investigation for comparison with isolations from animals, abattoirs and man, but is outside the scope of the present study.

METHODS

The technique used in Cardiff to isolate salmonellas from swabs has recently been described in detail (Harvey, Price, Foster & Griffiths, 1969). We shall, therefore, confine ourselves to general principles in this paper.

Drain and sewer swabbing is a useful means of salmonella surveillance. It is not as sensitive in abattoir investigation as examination of caecal faeces from slaughtered animals. In human surveillance it is more rewarding than relying on records of clinical food poisoning. It requires an efficient isolation technique.

Important technical factors are: (*a*) length of exposure of swabs to sewage; (*b*) enrichment; (*c*) selective media; (*d*) secondary enrichment; (*e*) search for multiple serotypes in single samples.

Length of exposure of swab to drain or sewage flow

Exposure may vary from mere wiping the swab along the drain or sewer surface (Harvey & Phillips, 1955), to leaving it in the flow for up to 7 days. The decision which method to use is administrative rather than technical. If rats are inclined to remove swabs, the wipe technique may often be valuable. If two visits to an area each week are not convenient, the 7-day period is useful. A recent survey on human sewage employed both techniques (Harvey *et al.* 1969).

A heavily soiled swab may require 48 hr. incubation in selenite F to produce a positive result (Guth, 1916; Leifson, 1936; Harvey, 1965), whereas a 'wipe' swab may produce a pure culture of salmonellas from a 24-hr. plating. We seldom subculture from selenite at 48 hr. nowadays as secondary enrichment from selective

agars has replaced this technique. In early sewer surveys, however, multiple subculture from selenite F broth was an integral part of salmonella isolation (Harvey & Phillips, 1955).

Enrichment

The usual fluid media chosen in this country are selenite F broth, balanced tetrathionate (Knox, Gell & Pollock, 1943), Kauffmann–Muller tetrathionate (Heard, Jennett & Linton, 1969) and the malachite green, magnesium sulphate broth of Rappaport, Konforti & Navon (1956). The selenite and tetrathionate broths can be made to function extremely well at 43° C. The malachite green medium has to be used at 37° C. In Cardiff we have for many years had a preference for selenite F broth + $1/10^6$ brilliant green (final concentration). This quantity of dye does not prevent the use of 43° C. for incubation. Commercial selenite brilliant green broth which has a higher concentration of dye may not allow such a high incubation temperature to be used. There is now reasonable confirmation from several parts of the world that incubation temperatures above 37° C. and not higher than 43° C. aid salmonella isolation. The subject has recently been discussed (Harvey & Price, 1968).

In Cardiff, we culture the entire swab in the jar in which it is received. It is merely covered with single strength enrichment medium and incubated at 43° C. This saves relabelling samples. We have always favoured culturing the whole swab. Large inocula in enrichment media usually benefit isolation (Harvey, 1965). Many workers, however, prefer the use of dilutions of swab fluid.

Any single enrichment medium, if inoculated with material containing two serotypes, may have a bias towards allowing more rapid multiplication of one of them. This problem is being currently studied on paired naturally infected samples. It is highly relevant to the conduct of unbiased surveys.

Plating media

The plating media favoured are: brilliant green MacConkey agar (Wilson & Blair, 1931; Harvey, 1956), Oxoid brilliant green agar, deoxycholate citrate agar, S.S. agar (Oxoid or Difco) and de Loureiro's (1942) modification of Wilson & Blair's bismuth sulphite agar. We find brilliant green MacConkey the best all round medium, closely followed by Oxoid brilliant green agar. Deoxycholate citrate agar is essential for optimum isolation of *S. dublin*. Wilson & Blair's medium is necessary for easy recognition of subgenus III salmonellas (Harvey, Price & Dixon, 1966).

Secondary enrichment

Growth is removed by a short throat swab from deoxycholate citrate agar plates, passed through a modified Craigie tube (Harvey & Price, 1967*a*) and subcultured to brilliant green MacConkey. Secondary enrichment is absolutely necessary in drain and sewer swab investigations if selenite F is used for primary enrichment. The combination of selective agars described here allows strains of *S. dublin* to be recovered. In the original description of the method, difficulty was experienced

with this serotype. The technique is also excellent for separating pseudomonas from salmonellas (Ino & Graber, 1955).

Search for multiple serotypes

Any drain swab is potentially contaminated with several salmonella serotypes. Although the number of serotypes isolated from a sample is a function of the number of colonies picked (Harvey & Price, 1967*b*), multiple picking will *not* always reveal some of the epidemiologically interesting serotypes. This was found in the isolation of salmonellas from Indian crushed bone. On one occasion 50 colonies were picked from an infected sample. Only one serotype was found. The serological technique described by Harvey & Price (1967*b*) allowed isolation of two further serotypes and this technique is now used as a routine. Unless employed, valuable information will be lost. The method has recently been adapted to the more specific isolation of *S. typhimurium* from Argentine bone (R. W. S. Harvey & T. H. Price, unpublished).

RESULTS

Animals at abattoirs

The original investigation of Cardiff abattoir lasted 3 years. Monitoring of this slaughter house continued, however, and only ceased with the closure of the premises in 1967. Drains were selected which gave some information on the type of animal bringing salmonellas into the abattoir. Clear-cut information was rarely obtained, but in a period of high incidence of *S. typhimurium*, phage type 12*a*, and *S. brandenburg*, positive results were obtained for many weeks in the drain receiving material from slaughtered pigs and not from the drain monitoring cattle and sheep only. In Barry abattoir and Pontypridd abattoir, it was possible to sample areas solely concerned with pig slaughter. In the former establishment, 20 % of swabs were positive over a 6-year period, in the latter, 30 % over 12 months. In a recent survey in the new Cardiff abattoir, 10 % of 1000 pig caecal faeces and 5 % of 1000 caecal swabs contained salmonellas. The amount of faecal material on a caecal swab is approximately 0·6 g. (J. Morgan, personal communication). Many serotypes found in pigs were also present in raw ingredients of locally distributed animal food.

The range of serotypes isolated in the years 1957–67, is given in Table 1 in historical order of isolation. This arrangement produces an inverted step-like effect and suggests the seeding of farm animals each year with new serotypes possibly from animal feed ingredients.

The isolation of Arizona 26:29–30 in 1965 is of interest. This serotype is pathogenic to sheep in Europe and has been isolated from human infections in sheep-herding tribes of Red Indians in the U.S.A.

It will be noted that *S. typhimurium* and *S. dublin* dominate the serotype pattern. The former, however, was not isolated for the first 20 months of the survey. Is the relative dominance of *S. typhimurium* in abattoirs of comparatively recent origin? *S. dublin* was not found in Cardiff abattoir in 1960–62. As *S. dublin* is endemic in South Wales, we suspect that this was due to a technical artifact and

that the increased number of isolations of *S. typhimurium* interfered with the recovery of *S. dublin*. A slight change in technique possibly contributed to the reappearance of *S. dublin* in 1963.

Table 1. *Cardiff abattoir*

(Range of serotypes isolated.)

Salmonella serotype	Year 1957–67											Other isolations
	57	58	59	60	61	62	63	64	65	66	67	
S. dublin	12	7	1	—	—	—	6	11	10	29	14	—
S. senftenberg	1	—	1	—	—	—	—	—	—	—	—	—
S. meleagridis	1	2	4*	—	—	—	—	1	—	—	—	—
S. anatum	3	—	4	—	—	1	—	—	—	—	1	—
S. bovis-morbificans	2	—	—	—	—	—	—	—	1	—	—	—
S. enteritidis	5*	1	1	2	—	—	1	1*	1*	1	1	—
S. thompson	4	—	1	—	—	—	—	1	—	—	—	—
S. muenchen	—	3	1*	—	—	1	—	—	—	—	—	—
S. kiambu	—	1	—	—	—	—	—	—	—	—	—	—
S. derby	—	4*	—	—	—	—	—	4	1	1	—	—
S. typhimurium	—	16*	31*	38*	3	13*	23*	18	10	24	12	—
S. kentucky	—	—	2	—	—	—	—	—	—	—	—	—
S. abony	—	—	1	—	—	—	—	—	—	—	—	—
S. weltevreden	—	—	1	—	—	—	—	—	—	—	—	—
S. newport	—	—	—	1	—	—	—	—	—	1	—	—
S. give	—	—	—	2	—	—	—	1	—	—	—	Pigs and butchers' equipment 1960
S. brandenburg	—	—	—	4	4	—	—	51*	4*	—	—	Butchers' equipment 1964
S. taksony	—	—	—	—	3	—	—	—	—	—	—	—
S. heidelberg	—	—	—	—	1*	4	—	—	—	—	—	—
S. paratyphi B, var. java	—	—	—	—	—	2	—	—	—	—	—	Type Battersea
S. luke	—	—	—	—	—	1	—	—	—	—	—	—
S. menston	—	—	—	—	—	—	1	—	—	—	—	—
S. agama	—	—	—	—	—	—	—	1	—	—	—	Local abattoir, man and butchers' equipment 1963
S. chester	—	—	—	—	—	—	—	2	—	—	—	—
S. bredeney	—	—	—	—	—	—	—	3	—	—	—	—
S. panama	—	—	—	—	—	—	—	3	—	—	—	—
S. poona	—	—	—	—	—	—	—	1	—	1	—	—
S. uganda	—	—	—	—	—	—	—	1	—	—	—	—
S. havana	—	—	—	—	—	—	—	—	5	—	—	—
Arizona 26:29–30	—	—	—	—	—	—	—	—	3	—	—	—
S. stanley	—	—	—	—	—	—	—	—	13*	3	—	—
S. takoradi	—	—	—	—	—	—	—	—	—	1	—	—
S. oranienburg	—	—	—	—	—	—	—	—	—	1	—	—
S. eimsbuettel	—	—	—	—	—	—	—	—	—	1	—	—
S. indiana	—	—	—	—	—	—	—	—	—	—	1	—

* Local infection in man due to same serotype or phage type in same year.

The remarks column in Table 1 amplifies information concerning certain serotypes. Asterisks denote the occurrence of human infections in the same year as an isolation from the abattoir—often within a few weeks.

Table 2. *Correlation of human and abattoir isolations*

(All Glamorgan abattoirs included.)

Serotype	Year of incident	Month 1	2	3	4	5	6	7	8	9	10	11	12	Abattoir isolations: Cattle drain	Pig drain	*Incidents England and Wales	*Rank of importance in England and Wales in year
S. meleagridis	1958	.	.	.	.	+	.	.	.	.	.	.	.	.	.	4	.
S. derby	1958	.	.	.	.	.	.	.	.	+	.	.	.	Sept. 1958	.	58	7
S. derby	1962	.	.	.	.	.	.	.	+	.	.	.	.	.	Sept. 1962	11	.
S. typhimurium, 1 var. 5	1959	.	.	.	.	.	.	.	.	.	+	.	.	Oct. 1959	.	.	.
S. heidelberg	1959	.	.	.	.	.	.	.	.	.	+	.	.	.	.	182	4
S. heidelberg	1961	.	.	.	.	.	.	.	.	.	+	.	.	.	Jan. 1962	289	2
S. typhimurium, 2 (12)	1960	.	.	.	.	.	.	+	+	+	+	.	.	July 1960	Sept. 1960	.	.
S. typhimurium, 2 (12*a*)	1962	.	.	.	.	.	.	.	.	.	+	+	+	.	Oct. 1962	.	.
S. typhimurium, 2 (12*a*)	1963	.	.	.	+	+	+	+	+	+	+	+	+	May 1963	April 1963	.	.
S. typhimurium, 4	1963	.	.	.	.	.	+	.	.	.	.	.	.	.	June 1963	.	.
S. agama	1963	.	.	.	.	.	.	+	.	.	.	.	.	.	July 1963	12	.
S. brandenburg	1964	.	.	.	.	.	+	+	+	+	+	+	.	.	.	312	2
S. stanley	1965	.	.	.	.	.	.	.	+	+	+	+	.	.	.	50	7
S. typhimurium, U 163	1967	.	+	.	.	.	.	.	.	.	.	.	.	Feb. 1967	.	.	.
No. of occasions same serotype or phage-type found in same month in abattoir and man		.	1	.	1	2	3	4	5	5	8	4	2	.	.	.	.

* Reports (1959), (1960), (1962), (1963), (1964*a*), (1965), (1966).

The pattern of salmonellosis in Cardiff abattoir is sometimes representative of national trends. This is demonstrated in Table 2 in which the occurrence of the same serotype or phage type in the same month in abattoir and in man is charted. Short term abattoir drain surveys fail to provide evidence of seasonal effects on environmental contamination. A long-term survey, however, will give information on such trends. This is illustrated in Table 3, which is arranged in quarters of the year. The third quarter shows maximum abattoir contamination. A seasonal effect on monthly correlations of serotypes isolated from abattoir and man is also evident in Table 2.

Table 3. *Cardiff abattoir*

(Swabs positive at different seasons of year, 1957–67.)

Quarter of year	Number of swabs examined	Number positive
January–March	280	83 (30)
April–June	246	95 (39)
July–September	282	150 (53)
October–December	273	127 (47)

Figures in parentheses are percentages.
Highest percentage of positive swabs is obtained in third quarter of year.

Table 4. *Cardiff abattoir*

(Annual incidence of positive swabs 1957–67.)

Year	Percentage of swabs positive	Ratio of ovine: bovine species
1957	33	3:1
1958	31	3:1
1959	59	6:1
1960	72	4:1
1961	11	4:1
1962	25	4:1
1963	44	4:1
1964	57	6:1
1965	57	7:1
1966	51	6:1
1967	54	3:1

Sheep are the dominant species in this abattoir. Changes in ratio between sheep and cattle show no correlation with percentage of swabs positive.

Table 4 records changes in annual incidence of positive swabs in the abattoir for the years 1957–67. It will be noted that the annual percentage of positive swabs varies considerably. There was no correlation between such changes and alterations in the ratio of sheep to cattle slaughtered. In Cardiff, sheep dominate the animals killed. Report (1964*b*) showed a correlation between abattoirs with high environmental contamination and the proportion of cattle slaughtered. In general, abattoirs killing a high proportion of cattle and a low proportion of sheep were heavily contaminated.

Meat wholesalers and retailers

Our main study of this point on the salmonella pathway has been conducted at Cardiff Central Market. This is a two-level structure comprising a gallery and ground floor. The gallery houses a pet meat stall and several stalls selling pet animals. Terrapins and tortoises are included in animals for sale. The ground floor consists of premises selling fish, fruit and vegetables, meat, poultry, dairy produce and flowers. Drains running along north and south aspects of the ground floor were

Table 5. *Cardiff covered meat market*

(Range of serotypes isolated.)

	Year 1963–68						
Salmonella serotype	63	64	65	66	67	68	Remarks
S. typhimurium	10	3	1	1	4	—	—
S. kingabwa	1	—	—	—	—	—	—
S. jerusalem	1	—	—	—	—	—	—
S. richmond	2	—	—	—	—	—	—
S. eastbourne	1	—	—	—	—	—	Prevalent in man 1963
S. derby	1	—	1	—	—	—	Infection in man 1965
S. dublin	—	1	—	—	—	—	—
S. clifton	—	1	—	—	1	—	Subgenus II
S. brandenburg	—	1	—	—	1	—	Prevalent in man 1964
S. oranienburg	—	1	—	1	—	—	—
S. senftenberg	—	—	1	—	—	1	—
S. paratyphi B, var. java, 1 var. 6	—	—	1	—	—	—	Outbreak in N. Wales 1959, 1964
S. bovis-morbificans	—	—	1	—	—	—	—
S. stanley	—	—	4	—	1	—	Prevalent in man 1965, 1967
S. enteritidis, essen	—	—	1	—	—	—	—
S. liverpool	—	—	—	2	—	—	Infection in man 1966
S. havana	—	—	—	1	—	—	—
S. panama	—	—	—	5	1	—	Infection in man 1967
S. abony	—	—	—	1	1	—	—
S. schwarzengrund	—	—	—	1	—	—	Infection in man 1966
S. bredeney	—	—	—	1	—	—	—
S. kiambu	—	—	—	1	—	—	—
S. indiana	—	—	—	4	—	—	Infection in man 1967
S. sofia	—	—	—	—	1	—	Subgenus II
S. newport	—	—	—	—	2	—	Infection in man 1967
S. sheffield	—	—	—	—	1	—	—
S. poona	—	—	—	—	1	—	—
S. javiana	—	—	—	—	1	—	—
Arizona 26:32–21	—	—	—	—	1	—	Subgenus III. Human infection, U.K. 1966
S. manhattan	—	—	—	—	1	—	—
S. reading	—	—	—	—	2	—	Infection in man 1967
S. livingstone	—	—	—	—	—	1	—

sampled over 6 years. The salmonella isolations from swabs placed in these drains are given in Table 5. The remarks column records information on certain serotypes considered relevant to their epidemiology. Certain salmonellas appear in Table 5 not found in Table 1 in the same time period (*S. eastbourne*, *S. liverpool*, *S. schwar-*

zengrund), although they were found in overt infection in man in the same year. The market sells poultry and imported meat. This could explain isolation of different serotypes from those found in the abattoir. The isolation of *S. paratyphi* B, var. java phage-type 1 var. 6, is interesting as this organism was responsible for sharp outbreaks of human infection in North Wales in 1959 and 1964. These outbreaks were probably from an animal source. The slime layer test is done as a routine on all strains of *S. paratyphi* B isolated from abattoirs and feeding stuffs.

Subgenus II strains found in this market may be due to the presence of animals carrying such organisms (tortoises and terrapins). Alternatively they may have gained entry to the U.K. in imported animal food from countries where this subgenus is common (Brede, 1964).

Table 6 records a parallel investigation on surfaces of butchers' equipment using broth moistened 'wipe' swabs (Harvey & Phillips, 1955). They were taken in a period of high incidence of human salmonellosis. The table is self explanatory and emphasizes danger of cross-contamination of meat in butchers' premises. The majority of samples were taken from three large wholesale butchers in Cardiff.

Table 6. *Equipment surfaces in retail and wholesale butchers sampled by 'wipe' swab technique June* 1963–*January* 1964

Surface found positive	Isolation
Sausage machine	*S. typhimurium*, type 12*a*
Fat trimming table (wood)	*S. agama*
Mincer	*S. agama*
Mincer	*S. agama*
Faggot preparation table (marble topped)	*S. typhimurium*, type 12*a*
Preparation table (wood)	*S. typhimurium*, type 12*a*
Inside surface of refrigerator	*S. agama*
Mincer	*S. agama*
Galvanized sink	*S. agama*
Unspecified surfaces (7)	*S. agama*: 5 isolations *S. typhimurium*, type 12*a*: 2 isolations

Total surfaces sampled = 305,
Isolations of *S. typhimurium*, type 12*a* from Man, June 1963–January 1964 = 41,
Isolations of *S. agama* from Man, June 1963–January 1964 = 1.

During this period *S. typhimurium*, phage type 12*a*, was isolated on 23 occasions from Cardiff abattoir and on 21 occasions from the pig area drain in Barry abattoir. Both establishments supplied meat to the wholesale butchers investigated. *S. agama* was found in the pig area drain in Barry abattoir in July 1963. The swab from which it was isolated also contained *S. typhimurium*, type 12*a*. The separation of these antigenically similar serotypes was made by the serological method described by Harvey & Price (1967*b*).

Man, sewage, sewage polluted water

Salmonellosis in man in our area is at a low ebb. The numbers of overt infections. diagnosed each year in Cardiff from 1963 to 1968 were: 160, 74, 80, 42, 111 and 57 As the medical importance of any salmonella focus in an area can best be judged

Table 7. *Serotypes isolated from human sewage*

Serotype	1967								1968						
	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	April	May	June	July
S. paratyphi B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. brandenburg	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
S. dublin	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
S. give	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. anatum	+	+	−	−	+	+	+	−	−	+	−	−	−	−	−
*S. kraaifontein**	−	+	−	−	−	−	−	−	+	−	+	−	+	−	−
S. enteritidis	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−
S. typhimurium	−	+	−	−	−	−	−	+	−	−	−	+	+	+	−
S. oranienburg	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
S. schwarzengrund	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
S. stanley	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−
S. manhattan	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−
S. newport	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−
S. panama	−	−	−	+	+	−	+	+	−	+	−	+	−	+	+
S. bredeney	−	−	−	−	+	+	−	−	−	−	−	−	−	+	−
S. indiana	−	−	−	−	+	+	+	+	+	−	−	−	−	+	−
S. senftenberg	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
S. derby	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
S. galiema	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
S. kentucky	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
S. livingstone	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
S. fischerkietz	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
S. duisburg	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
*S. bleadon**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
*S. uphill**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+

The sewage was derived from an estate of 4000 persons. Neither industry nor butchers' shops were located in the area investigated. Surface water was found to be negative for salmonellas.

* Subgenus II.

by its impact on man, it was thought necessary to examine local human salmonellosis by more sensitive means than mere records of clinical infection.

From several years examination of samples of the river Taff, which drains a densely populated part of North Glamorgan, we came to the conclusion that overt salmonella infection represented only a very small proportion indeed of total human infection in the area. It was essential to be certain that river isolations were not coming from industrial effluent. In 1967, therefore, we began a detailed examination of human sewage from an estate of 4000 persons (Harvey *et al.* 1969). The use of selected sewage examination had previously been of value in the bakery survey (Harvey & Phillips, 1961).

The estate was purely residential. It was situated on a hillside where sewage back flow was impossible. The sewage from other residential areas could not possibly enter the drainage investigated. It contained neither industry nor retail butchers' shops. Parts of the area contained no shops at all. Samples of surface water were negative for salmonellas. Samples of sewage from the main drain of the estate were consistently positive. Points were chosen spreading back into the branches of the sewerage system. One sampling point took sewage from 1000 persons. The results of the survey covering the period May 1967–July 1968 are presented in Table 7. From the sampling point monitoring 1000 people, 12 different serotypes were isolated over a year. This gives some idea of minimum human infection/1000 p.a. If we exclude *S. paratyphi* B, salmonellas were isolated from the estate on 44 of 54 sampling occasions. These organisms, therefore, regularly reach man. The serotype range was wide and several exotic species were isolated, suggesting that salmonellosis in man sometimes starts outside the United Kingdom. Subgenus II strains were occasionally found. It would seem that a frequently infected and regularly consumed vehicle carries salmonellas into households. We consider poultry and pig products possible sources of infection. These animals frequently consume infected feed.

DISCUSSION

Drain swabbing in Cardiff covered the years 1955–69. The early part of this period witnessed a major change in epidemiological attitudes to salmonellosis. Realization came, very gradually, that contaminated food ingredients were sources of infection of greater importance than human excreters. This point was not made without opposition. It was largely due to the work of Thomson (1953). The use of drain swabs served to emphasize this (Harvey, 1957). By this means we were able to chart entry into, persistence in and exit of serotypes from situations such as bakeries, abattoirs, knackers' yards, butchers, retail markets and human sewage.

The development of phage-typing of strains of *S. typhimurium* by Anderson & Wilson (1961) immensely increased accurate comparison of veterinary and human isolations. *S. typhimurium* is by far the most important salmonella species common to man and animals. Comparative studies became of vital importance with the demonstration of transferable drug resistance (Anderson & Lewis, 1965; Anderson,

1968). The drain swab technique increased opportunities for contrasting food, animal and human salmonellosis.

Experience suggests that the abattoir drain swab is an economical check on appearance and continued persistence of a serotype in a slaughter house. Persistence is not infrequently followed by infection of man (Table 2). Warm weather periods are particularly dangerous, and forewarning of a build-up of infection could allow application of intensive hygienic measures.

It has been asked if sewer swabbing could be of value in an epidemic to clarify the origins and means of spread of infection. We do not think that this technique, *per se*, can help greatly to control an established outbreak. We believe that proper interpretation of results already obtained may ultimately serve to check the passage of salmonellas from animal to man. We consider it logical to think of the origin of salmonellosis in terms of the initial or, at least, a remote origin. An epidemic caused by what is termed an exotic serotype has an exotic source. The finding of an outbreak serotype in a butcher's shop environment is surely evidence of mode of spread (Harvey *et al.* 1963). The clustering of human infections round a series of shops shown to have the relevant serotype on the premises indicates advisability of hygiene checks in the area. This is not beyond the powers of health authorities.

The discovery that a wide range of salmonellas frequently reach man (Harvey *et al.* 1969), suggests that vehicles frequently infected with many serotypes bring infection into households. Such vehicles must be food that is commonly consumed. The increased sale of broiler chickens has recently been recorded (Gould & Rhodes, 1969). These animals are of interest in that they are frequently fed on infected animal feed.

It is usually maintained that, as *S. typhimurium* and *S. dublin* are seldom found in animal feed ingredients, feeding stuffs play little part in transmission of these two serotypes. This may be so. Few would deny, however, that these two species are frequently found in animal intestines on the way from abattoir to rendering plants concerned with manufacture of products destined for animal food. How do we reconcile this with their comparative rarity in home produced meat and bone meal? By careful technique, *S. typhimurium*, phage-type 32, was found in 4/12 samples of bone meal associated with a large outbreak of food poisoning in Scotland. The Cardiff laboratory was responsible for three of those isolations. Isolating *S. dublin* in the presence of more vigorously growing salmonella species is not always easy. This serotype can be easily inhibited by brilliant green, it is poorly isolated on Wilson & Blair (1931) medium and the use of tetrathionate, commercial selenite brilliant green and malachite green magnesium sulphate enrichment media can often prevent its isolation. Selenite F broth subcultured to deoxycholate citrate agar is the optimum means of recovery. Epidemiological interpretation of failure to isolate this serotype must be treated with reserve.

We should like to acknowledge the help given by Professor Scott Thomson, and the technical assistance of Mr T. R. Liddington, Mr John Morgan and Miss Sheila Grant.

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J. Hyg., Camb. (1970), **68**, 625
Printed in Great Britain

The laboratory transmission of Coxsackie A6 virus by mosquitoes

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(*Received* 25 *June* 1970)

SUMMARY

Coxsackie A6 virus, strain V29, originally isolated from *Aedes polynesiensis* in Fiji, was found to survive in *A. australis* for 5 days after intrathoracic injection and for 6 days after feeding on viraemic mice, and in *Culiseta tonnoiri* for 8 days after feeding.

The virus level in both fed and injected mosquitoes fell steadily after infection and never exceeded the original level.

No virus transmission was obtained in 46 successful second feedings on days 10–14 with *A. australis*, but three transmissions were obtained with *C. tonnoiri*, two on day 10 and one on day 13, from 12 successful second feedings.

INTRODUCTION

The isolation of nine strains of Coxsackie A6 virus from *Aedes* (*Stegomyia*) *polynesiensis* Marks in Fiji at a time when human Coxsackie infections were known to be occurring (Maguire & Macnamara, 1966) indicated that mosquitoes might be potential vectors of enteroviruses in nature, and that in certain areas, vector transmission might play a small, but significant, part in the epidemiology of such infections. Several authors have reported the isolation of enteroviruses from flies and other insects (reviewed by Gelfand, 1961), but there have been very few studies on the multiplication or survival of enteroviruses in insects, or on the ability of blood-sucking arthropods to transmit enteroviruses by bite. One brief report has been published on the ability of mosquitoes to support the multiplication of Coxsackie viruses (Taylor, 1955).

In this study, investigations have been made into the survival of Coxsackie A6 virus in two species of mosquito, and into the transmission of the virus to normal newborn mice by mosquitoes infected by intrathoracic injection of virus or feeding on viraemic newborn mice.

MATERIALS AND METHODS

Virus

V29, one of the original Fiji strains of Coxsackie A6, was used at the second and third mouse passage levels. Virus suspensions for intrathoracic injection of mosquitoes were prepared from fresh newborn mouse brains harvested 72 hr. after

intraperitoneal (i.p.) injection. Suspensions were made by grinding the brain in a mortar, adding diluting fluid containing calf serum and antibiotics, and clearing the suspension by spinning at 2000 rev./min. for 5 min. The mice, all random-bred albino stock, were inoculated i.p. within 24 hr. of birth and usually developed a viraemia of over $10^{4\cdot5}$ LD 50/0·02 ml. by the 48th hr. after infection and over $10^{6\cdot0}$ LD 50/0·02 ml. by 72 hr. when deaths began to occur.

The virus titres in individual mosquitoes and mouse bloods were determined by inoculating 24-hr.-old newborn mice intracerebrally (i.c.) and i.p. simultaneously.

Mosquitoes

Laboratory-reared *Aedes* (*Halaedes*) *australis* Erichson and field-trapped *Culiseta* (*Climacura*) *tonnoiri* Edwards were used in this study. *A. polynesiensis* does not occur in New Zealand.

After feeding or injection, infected mosquitoes were kept under controlled conditions of humidity and temperature (mean 20° C.) in an insectory. Any mosquitoes dying and others taken for virus assay at various intervals after infection were frozen at − 34° C.

Intrathoracic injection of mosquitoes

Mosquitoes were lightly anaesthetized with carbon dioxide and then inoculated by injecting virus suspension into the thorax using a Pyrex microcapillary pipette. The average amount of virus injected was calculated by titrating suspensions prepared from several individual mosquitoes, frozen immediately after injection.

Feeding experiments

Viraemic newborn mice were attached to adhesive tape on wooden blocks which were then placed in cages of mosquitoes. The mice were relatively immobile and could not move sufficiently to discourage probing by the mosquitoes. After several hours at 32–35° C., the mice were removed and bled for virus assay, and engorged mosquitoes were removed and placed in individual glass vials containing moist cotton-wool covered with filter paper and capped by a circle of mosquito netting. The mosquitoes were kept alive by feeding occasionally with fresh apple slices and by placing a plug of cotton wool soaked in weak sugar solution on top of the netting.

Transmission

Attempts were made, at various intervals after engorgement or injection, to induce the infected mosquitoes to feed on normal 24-hr.-old mice to see whether or not transmission of the virus could be obtained. The mice were placed on top of the mosquito net cap covering the vial and they were held in place by a second piece of netting placed over them and secured loosely to the vial by a rubber band. The mice were thus immobilized without causing them any discomfort and yet they were directly accessible to the mosquito. To encourage feeding, the mosquitoes were deprived of food and water for 2 or 3 days before exposure to the mice. While this practice increased the death rate quite markedly, it did induce feeding,

Table 1. *Survival of Coxsackie A 6 virus in mosquitoes*

Species of mosquito	Infection method	No. of mosqs.	Geometric mean dose /mosq.	Days after infection										
				0	1	2	3	4	5	6	7	8	9	10 or >
Aedes australis	Injection	78	$10^{3\cdot5}$ LD 50	25/26*	3/20	NT	4/10	0/10	1/7	0/5	NT	NT	NT	NT
	Feeding	113	$10^{4\cdot5}$ LD 50	20/20	3/3	6/7	NT	3/3	1/2	1/3	0/8	NT	0/9	0/58
Culiseta tonnoiri	Feeding	126	$10^{5\cdot2}$ LD 50	7/7	1/1	12/12	16/18	6/6	2/2	6/7	NT	3/3	0/1	0/69

NT = No test done. * Mosquitoes containing virus/number of mosquitoes tested.

Table 2. *Titre of Coxsackie A 6 virus in mosquitoes at intervals after feeding or injection*

Species of mosquito	Infection method		Days after infection								
			0	1	2	3	4	5	6	8	10
Aedes australis	Intrathoracic injection	No. tested	6	4	NT	3	3	2	NT	NT	NT
		Mean log. titre/mosq.	3·5	2·6	—	0·4	< 0·2	< 0·2	—	—	—
	Feeding	No. tested	6	3	3	NT	3	2	3	NT	3
		Mean log. titre/mosq.	4·5	3·3	2·1	—	0·6	0·3	< 0·2	—	< 0·2
Culiseta tonnoiri	Feeding	No. tested	7	1	3	3	3	2	3	3	2
		Mean log. titre/mosq.	5·2	4·6	3·9	3·6	2·7	1·9	1·7	1·1	< 0·2

NT = No mosquitoes tested.

especially with the delicate *C. tonnoiri*. Any mosquitoes which did take a second meal, or which were observed to probe for any length of time, were immediately frozen for subsequent virus assay and the mice were marked by tattoo for identification purposes.

Virus identification

Virus recovered from mice bitten by infected mosquitoes was identified using a mouse neutralization test incorporating a Coxsackie A6 antiserum of known potency.

RESULTS

Virus survival

The number of mosquitoes containing detectable virus at various intervals after feeding or injection is shown in Table 1. Table 2 shows the amount of virus in selected mosquitoes at intervals after infection.

Transmission

Table 3 shows the number of mosquitoes exposed at intervals to uninfected mice and the number of second feedings and transmissions obtained.

Table 3. *Transmission studies with Coxsackie A 6 in mosquitoes*

Species of mosquito		Days after first feeding							
		3	6	9	10	12	13	14	16
Aedes australis	Number of infected mosquitoes exposed to mice	44	40	38	38	30	32	40	NT
	Successful second feedings	0	0	0	8	18	8	12	—
	Death in exposed mice	0	0	0	0	0	0	0	—
Culiseta tonnoiri	Number of infected mosquitoes exposed to mice	NT	NT	30	24	21	20	18	16
	Successful second feedings	—	—	0	4	3	5	0	0
	Deaths in exposed mice	—	—	0	2*	0	1*	0	0

* No virus was detected in the mosquitoes biting these mice. Virus recovered from the mice was identified by neutralization test.
NT = No mosquitoes exposed.

DISCUSSION

Coxsackie A6 survived longer in *A. australis* mosquitoes which had had a blood meal than in those which had been infected by injection, and *C. tonnoiri* engorged on mouse blood harboured the virus longer than did the engorged *A. australis*. Both these observations could be explained on the basis of differences in initial doses.

It is not surprising that Coxsackie A 6 is capable of surviving for up to 8 days in mosquito tissue since, like most enteroviruses, this virus is relatively stable over a wide range of temperatures, pH and chemical environment (reviewed by Dalldorf & Melnick, 1965). There was no evidence of active multiplication of the virus and at no time did the titre in the mosquito exceed the amount originally ingested or injected.

Transmission of Coxsackie viruses by mosquitoes is theoretically possible since enteroviruses may be present in the blood of infected persons for one or more days in the early stages of certain infections and virus has been isolated from the blood of such persons on many occasions (Yoshioka & Horstmann, 1959; Gear, 1961–2; Rodrigues *et al.* 1964; Maguire & Macnamara, 1966). It is likely that many more isolations would have been reported but for the fact that the viraemic phase is probably over before the onset of symptoms (Yoshioka & Horstmann, 1959). A blood-sucking arthropod feeding at this critical period could well act as a vector by biting a second uninfected individual even in the absence of active virus multiplication in the mosquito tissue.

The transmission studies were complicated by the fact that mosquitoes generally do not take a second blood meal during the period after infection when virus was shown to be present. *A. australis* is a rather hardy species which survived handling well and which could be readily induced to take a second blood meal on or after the 10th day after the first meal. In one experiment, 13 of 22 *A. australis* exposed to new uninfected newborn mice on day 12 took a second full blood meal, but no transmissions were obtained. This would suggest that these mosquitoes may not be very efficient vectors of Coxsackie viruses in nature. On the other hand, *C. tonnoiri* is a very fragile species which did not survive well in the laboratory and which could only be induced to feed a second time with some difficulty. The transmission of virus by *C. tonnoiri* on days 10 and 13, when there was no detectable virus in the whole ground-up insect, suggests that i.p. plus i.c. inoculation of suckling mice used to detect virus in the mosquito tissue may not be as efficient a method of infecting as inoculation by mosquito bite. Subsequent comparative titrations of Coxsackie A 6 in mice using the combined i.c./i.p. route or the subcutaneous route showed that the subcutaneous route was slightly more sensitive as a method of detecting virus, the mean difference in titre being $10^{0.8}$ LD 50. The possibility that the suspension of mosquito tissue itself was absorbing or inactivating the virus was also tested and it was found that less than $10^{0.5}$ LD 50/ml. drop in titre occurred when Coxsackie A 6 was incubated with a mosquito suspension for 1 hr. at 37° C.

With a small number of successful transmissions such as those described above, account must be taken of the possibility that the virus recovered from the newborn mice could have arisen from contamination from some other source. In this case it would be very unlikely that such contamination did occur since virus was only recovered from those mice which were observed to have been bitten by infected mosquitoes, and no other work was being done with Coxsackie A 6 in the laboratory at the time.

The survival of the virus in a transmissible form, either within the mosquito or

as contamination of the mouth parts, for up to 13 days after feeding indicates that mosquitoes could play some role in the epidemiology of Coxsackie infections in areas of high mosquito population. However, in order to act as an efficient vector, the mosquito would have to bite a second time within a fairly short period.

The original Fiji isolations were all made from *A. polynesiensis*, although nine other species were also captured and tested at the same time. It seems therefore that species may differ in their ability to harbour the virus. This is supported by the present study where *A. australis* did not transmit, whereas *C. tonnoiri* did, even though considerably fewer second feedings were obtained with the latter.

The effect of temperature on the survival and transmission of Coxsackie A 6 has not been studied here. The two species of mosquito used failed to survive at temperatures much above 20° C. However, it is quite possible that virus multiplication could occur at the higher temperatures of the area of Fiji where the original isolations were made (mean summer temperature for Suva is 26° C.), and this possibility deserves further study. Such temperature effects on multiplication have been observed with arboviruses (Davis, 1932; Chamberlain & Sudia, 1955).

This work was supported by the Medical Research Council of New Zealand. I am grateful to Dr J. S. Pillai for his help with the supply of mosquitoes and to Mrs M. M. Prescott and Mr J. K. Scott for technical assistance.

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Printed in Great Britain

The natural history of molluscum contagiosum in Fijian children

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(*Received* 3 *July* 1970)

SUMMARY

A 10-month follow-up of 14 Fijian children suffering from molluscum contagiosum showed that the condition could last from about 6 months to about 3 years, but that any one lesion was present for only 2 months. There could be temporary relief from new lesions for at least 2 months; when complete remission occurred, it could be comparatively rapid. It is suggested that individual lesions should not be treated, in order to avoid scarring and because of their relatively rapid disappearance.

INTRODUCTION

Molluscum contagiosum is a lesion affecting human skin, and occasionally conjunctiva only, and is manifested as discrete waxy papules. Each lesion starts as a small sessile papule, smooth to the touch. It progresses in typical cases to form a slightly pedunculated lesion of a firm rubbery consistency up to 5 mm. in diameter. When resolution occurs, this typical lesion becomes umbilicated and shrinks to become rough to the touch, not unlike a developing or resolving verruca vulgaris lesion.

In 1967 the present author, in association with Postlethwaite and others of Aberdeen, published a paper to show that the causative virus in Fijians and in Aberdeen was identical, that the peak for ages of onset in a series of Fijians was 2–3 years, and in Aberdeen was 10–12 years, and that in Fijians lesions were commonest on the limbs, but in Aberdeen they were more common in the axilla.

METHODS

The previous paper (Postlethwaite *et al.* 1967) was unable to throw fresh light on the natural history of molluscum contagiosum lesions, so in 1968 the opportunity was taken to establish baselines for a longitudinal survey of a group of Fijian children suffering from the disease. During the course of a community health survey of a group of Fijian villages in the Lomanikoro area, 20 miles from Suva, the capital city, 17 cases were diagnosed as having the disease, of whom 13 were followed at approximately 10-week intervals for 10 months and a 14th for 8 months. Only these 14 will be considered in this report.

FINDINGS

On admission to the investigation the mean age of the 14 children was 5 years, ranging from 3 years (3 children) to 11 years (1 child). The mean number of lesions was 11, with a range from 1 to 27 and a distribution similar to that described in the previous paper. The mothers of the children, or other close adult relatives, were interrogated by Mr (now Dr) Halvor Brandt of Aberdeen and an estimate obtained as to the duration of the lesions. This gave a range from 2 weeks to 2 years, with a mean of 8 months.

Four of the 14 cases were considered to have been followed to the stage of cure, no new lesions having appeared for 4 months or longer. When the stated duration of the lesions was added to the period of observation, the mean duration of the lesions was 16 months, with a range from 6 to 28 months. However, in the other 10 cases the same calculations showed the same mean duration, but with new lesions still appearing; the longest duration was in the 11-year-old who was said to have had the condition for 2 years before the beginning of the investigation and whose lesions had dropped in number from nine at the beginning to one (new) lesion 10 months later. It would appear to be not unreasonable to expect a range of 6 months to 3 years for the duration of the condition.

Observations on the duration of individual lesions showed that each one was present for only 2 months or less. An area of skin would be clear at one inspection, contain a lesion at the next and be clear at the next. Since natural resolution of a lesion proceeds without scarring, while previous investigations had shown that any interference is followed by scarring, it would seem reasonable to recommend that lesions should not be treated. In two of the children, all lesions had disappeared at one visit but new ones were present at the next visit, suggesting that final spontaneous resolution should not be prognosticated until at least 4 months have passed without new lesions appearing. A large number of lesions at one period of time did not necessarily correlate with a prolonged duration of lesions. One child's lesion counts were: 23, 37, 15, 5, 1, Nil; the original duration of lesions was reported as being 5 months. In another child, with a history of lesions for 2 years, the lesion counts were 24, 3, 7, Nil, Nil, Nil.

The author would like to thank Dr C. H. Gurd, C.B.E., Director of Medical Services, Fiji, for permission to publish this article, and Dr Halvor Brandt, formerly of Aberdeen Medical School, for his assistance in obtaining case histories from relatives and in mapping the original lesions.

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J. Hyg., Camb. (1970), **68**, 633
Printed in Great Britain

The effect of a changed environment on bacterial colonization rates in an established burns centre

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(*Received* 6 *July* 1970)

SUMMARY

In an established burns centre which moved from an old building to new purpose-designed premises, colonization rates of patients' burns with *Staphylococcus aureus*, *Pseudomonas aeruginosa* and other Gram-negative bacilli were not reduced. Colonization rates with *Streptococcus pyogenes* increased but the increase was mainly due to multiple importations in the new premises of a strain of higher communicability than any seen in the old.

In the first 32 months in the new environment 10 patients were found colonized with pseudomonas on admission and 20 became colonized in the unit. A much higher proportion of patients with burns of more than 30% body surface became colonized than of patients with less. About one-third of the above 20 patients became colonized with strains already isolated from another patient; all but one of them had small area burns. Cross-infection was not observed from numerous heavily colonized patients with high percentage burns. This paradox is discussed in detail. Basin outflows in the new premises became colonized with *P. aeruginosa* of two serotypes not found on patients in this unit.

INTRODUCTION

The transfer of an established burns centre to a new purpose-designed building without change in the senior staff, the principles and practice of treatment, the range of patient intake and the frequency and technique of bacteriological monitoring enabled observation of the effect of an improved environment alone on the cross-infection rates. The old unit was housed in a war-time hutted building with a wide passage opening into four wards accommodating 7, 3, 3 and 3 patients, a total of 16. There was no air-conditioning. The new unit is built on the race-track principle and has four double and thirteen single rooms, accommodating a total of 21 patients. It is fully air conditioned on a high-velocity dual duct system using 100% fresh air. Patients' rooms are under a positive pressure with ten inlet changes of air per hour and six extract changes. The treatment rooms are at a higher rate of twelve inlet and twelve extract changes. Average working temperatures are 68° F. and average humidities 55%. Air enters rooms at ceiling level near the perimeter wall above patients' beds and leaves partly through extract filters in toilets and partly through pressure relief grilles into the corridors whence it is extracted at high level. The object is to minimize the risk of air-borne infection

to the patient by preventing air entry from passages to rooms. Repeated observations with titanium chloride smoke have not shown air-flow into patients' rooms even while opening their doors.

The régime in use from 1963, when detailed bacteriological records were started, was aimed specifically against the development of infection with *Pseudomonas aeruginosa*. It consisted of strict insistence on keeping burns dry, of minimal use of antibiotics, of exposure of burns in the early stages and of dressing in later stages with gauze squares impregnated with polyethylene glycol and 1 % nitrofurazone. Because of its success under the conditions in this centre both in preventing invasive infection and in preparation for grafting, it has been practised with little or no variation until the present time.

The clinical consequences of colonization with *Streptococcus pyogenes*, *Staphylococcus aureus* or *P. aeruginosa* were generally slight. Colonization with *Strep. pyogenes* was often associated with characteristic erythema, exudate and pyrexia which responded rapidly to benzylpenicillin or phenoxymethyl penicillin. The frequent additional presence of pencillinase-producing staphylococci, though it may have prolonged surface carriage, did not appear to prejudice clinical effectiveness. Erythromycin was sometimes used in place of penicillin. Antibiotic treatment of staphylococci or pseudomonas was seldom required. Tetracycline, ampicillin, methicillin, cloxacillin, carbenicillin, parenteral or topical gentamycin, sulphamylon or silver nitrate were seldom used. Prophylactic penicillin or erythromycin were not routinely used. Data on the proportion of patients with extensive burns, on which colonization rates with pseudomonas are strongly dependent, are given in the appropriate section.

METHODS

Swabs were taken as soon as possible after admission from one or more areas of burn and were repeated weekly or as thought necessary for clinical reasons. Each swab was cultured on a tryptic digest blood agar plate with 2·5 % agar. Single colonies from minority populations were subcultured either from zones of inhibition round antibiotic disks placed in the dense part of the inoculum or from the thin end of the spread, for identification by standard methods. Swarming of proteus was not a problem under these conditions. In the new unit nasal and throat swabs were cultured from patients on admission and from staff during periods of high streptococcal prevalence or on the appearance of a new strain of *Strep. pyogenes* other than an obvious importation.

Swabs from basin outflows for pseudomonas were inserted into the water traps a foot below the outlet. These and other environmental swabs were cultured on nutrient agar and in peptone water. Doubtful mixed growths were subcultured on 0·03 % cetrimide agar.

Strains of *Strep. pyogenes* were serotyped at the Public Health Laboratory, Oxford, by T-agglutination and M-precipitation typing (Williams & Maxted, 1953; Williams, 1958). Strains of *P. aeuruginosa* were typed at the Cross-Infection Laboratory, Colindale. Primary subdivision was by serological typing (Habs, 1958) with additions as specified in Lowbury *et al.* (1970). Individual serotypes were

further subdivided by phage typing; strains were considered different only when phage typing patterns were quite distinct.

Air sampling was done by the Safety Section of the Microbiological Research Establishment, Porton.

RESULTS

Air contamination rates

Slit sampling was done at a rate of 1 cu.ft./min. and plates were incubated for 21 hr. at 37° C. In the old unit 160 cu.ft. were sampled on four different occasions in the passages, yielding an average particle count of 14/cu.ft. with a maximum of 78. Corresponding observations in the passages of the new unit on 110 cu.ft. on four occasions yielded an average particle count of 1·6/cu.ft. with a maximum of 4·5. A further sample of 45 cu.ft. during electric polishing and other human activity yielded an average of seven particles/cu.ft., corresponding with the average air-contamination rate in a plenum ventilated theatre with much staff activity (Williams, Blowers, Garrod & Shooter, 1966). During dressing sessions in a patient's room sampling yielded maximum counts of 27 particles/cu.ft. and in a treatment room of 16 particles/cu.ft. When activity ceased counts rapidly returned to minimum level. During a period of high streptococcal prevalence in the new unit 0·6 particles/cu.ft. of *Strep. pyogenes* was grown from air samples in the passages.

Colonizations with Pseudomonas aeruginosa

Analysis of cultures from patients were made in three periods, for 21 months in 1963–4, for 16 months in 1966–7 up to the time of transfer to the new environment and for the first 32 months in the new unit, 1967–9. The findings are shown in Table 1 from which it is evident that the rates in each period are strikingly constant both in the number of patients already colonized on arrival and in those whose colonizations developed after admission. There was no reduction in colonizations in the new environment.

Table 1. *Comparison between the old and new units of patients with burns colonized by* Pseudomonas aeruginosa

	Months	Patients	Numbers colonized: Imported	Numbers colonized: Developed in unit
Old 1963–4	21	208	6 (2·9 %)	11 (5·3 %)
Old 1966–7	16	154	5 (3·2 %)	11 (7·1 %)
Total old	37	362	11 (3·0 %)	22 (6·1 %)
New 1967–70	32	351	10 (2·9 %)	20 (5·7 %)

Patients with extensive burns become colonized with pseudomonas much more often than those with smaller burnt areas. The 208 patients in the first period in the old unit (1963–4) and the 351 patients in the new unit (1967–9) were subdivided according to their percentage burns into three categories, 30 % or more of body surface, 15–29 and 14 % or less, excluding those, mostly old people with very high percentage burns, who died within a week of admission, and those already colonized on arrival. The findings are shown in Table 2, and the figures show clearly that

in terms of percentage burns, both the patient intake and the colonization rates were quite constant between 1963–4 in the old unit and 1967–9 in the new. They also show much higher colonization rates in patients with 30% or more burnt areas than in patients with less.

Table 2. *Comparison of colonizations with* Pseudomonas aeruginosa *according to percentage body surface burnt, in three categories between a period of* 21 *months in* 1963–4 *in the old unit and* 32 *months in* 1967–9 *in the new*

(Patients who were admitted already colonized or who died within 1 week of admission are excluded.)

Burn category	Environment	Number of patients	Number colonized with *P. aeruginosa*
30% or over	Old	8 (4% of total)	3 (38%)
	New	18 (5% of total)	7 (43%)
15–29%	Old	36 (18% of total)	4 (11%)
	New	58 (17% of total)	4 (7%)
14% or under	Old	154 (78% of total)	4 (3%)
	New	259 (78% of total)	9 (3%)

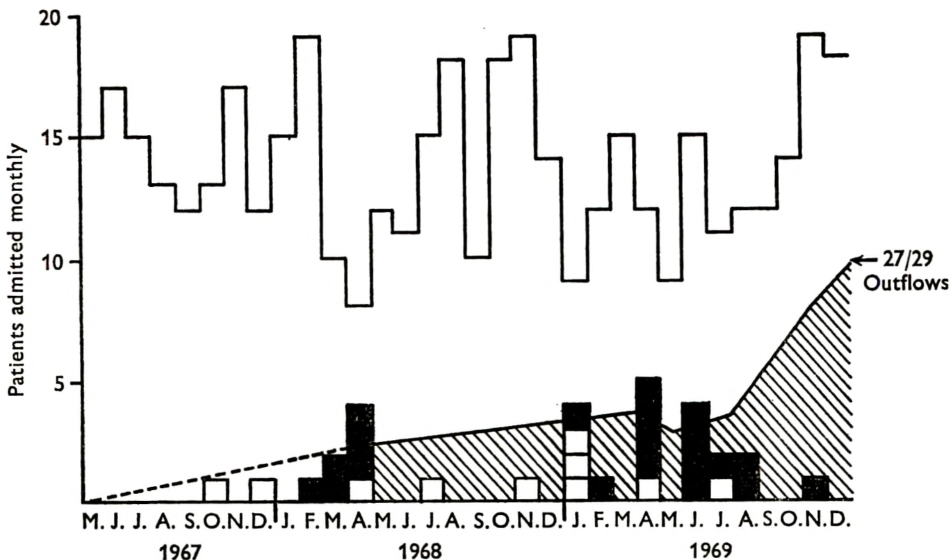

Fig. 1. New unit—first 32 months. □, Colonization with *P. aeruginosa* imported on burns (10 patients); ■, colonization with *P. aeruginosa* developing after admission (20 patients). Shaded area shows proportion of 29 basin and bath outflows colonized with *P. aeruginosa*.

Analysis of strains from patients in the new unit

Colonizations with *P. aeruginosa* in the first 32 months in the new unit were few enough to attempt to trace the source and spread of each type. If the 30 colonizations, 10 imported and 20 developing after admission, are plotted chronologically (Fig. 1) the latter are seen to be clustered in groups, six in February–April 1968, six in January–April 1969, seven in June–August 1969 and a single one in November

1969. These groups may be considered as separate incidents. Typing of the strains and other details are shown in Table 3.

Patients 3–8. Patient 1 had a different serotype to any of these six and patient 2, whose strain was not typed, had been discharged 12 days before patient 3 became colonized. Patient 5 was sharing a double room with patient 4 and was undoubtedly cross-infected from him. Patient 7 though not in direct contact with patient 6 shared an identical strain. Judged by phage patterns and serotypes, therefore, four patients were infected with different strains and the other two may be considered as cases of cross-infection.

Table 3. *Types of* Pseudomonas aeruginosa *colonizing the burns of* 30 *patients in the first* 32 *months in the new unit*

(Markedly different phage patterns are each represented by a letter as an aid to clarity. NT = not tested.)

Patient's number	Date first cultured	Days after admission	Weeks cultured	Phage pattern	Serotype
1	9. x. 67	Imported	2	A	11
2	28. xii. 67	Imported	5	NT	NT
3	12. ii. 68	15	5	B	6
4	9. iii. 68	9	3	C	6
5	30. iii. 68	64	4	C	6
6	1. iv. 68	10	1	D	2B
7	20. iv. 68	35	2	D	2B
8	24. iv. 68	5	2	E	5C
9	29. iv. 68	Imported	1	B[1]	6
10	15. vii. 68	Imported	3	F	10
11	30. xi. 68	Imported	2	G	4
12	8. i. 69	Imported	1	H	11
13	9. i. 69	Imported	1	H	11
14	20. i. 69	Imported	3	NT	NT
15	26. i. 69	31	2	J	3
16	8. ii. 69	8	16	K	6
	2. vi. 69			L	8
17	14. iv. 69	17	4	M	6
18	24. iv. 69	32	5	M	6
19	14. iv. 69	Imported	2	L	8
20	17. iv. 69	2	7	N	6
21	26. iv. 69	19	1	O	5D
22	12. vi. 69	3	6	P	5C
23	23. vi. 69	14	3	Q	—
24	25. vi. 69	16	4	R	3
25	28. vi. 69	3	1	S	5C
26	28. vii. 69	Imported	5	T	11
27	3. viii. 69	5	2	C	6
28	5. ix. 69	23	1	T[1]	11
29	14. ix. 69	27	1	T	11
30	24. xi. 69	24	1	U	3

Patients 15–18, 20 *and* 21. The strain from patient 14 was not typed but it shared a characteristically deep pigment with that from patient 15 and could have been the same. No source was evident for the strains on patients 16 and 17. Patient 18 was probably cross-infected from patient 17. The second strain isolated late from

patient 16 could have been derived from the strain imported on patient 19. The up-growth on patient 20 of the common serotype 6 as early as the second day is more consistent with carriage before arrival than of cross-infection from the strain of patient 16, the phage pattern of which might have undergone alteration after 10 weeks carriage. The strain infecting patient 21 had not been seen before. In this group there appear to have been three, perhaps four instances of cross-infection.

Patients 22–25, 27, 28 *and* 29. Patients 22–24 were among four North Africans burnt in an oil refinery accident, flown to England and admitted to the unit together. It is notable that these three whose burns became colonized with pseudomonas on the 3rd, 14th and 16th days were each infected with one of three unrelated strains. They are considered in more detail in the discussion. Patient 25 was colonized when patient 22 had been infected with the same serotype for 16 days, but the phage patterns were quite distinct. No source was evident for patient 27. The strain from patient 29 was identical with the imported strain from patient 26 and the strain from patient 28 might have been a variant from it. In this group there were one probable and two possible instances of cross-infection.

Patient 30. No source was evident.

Thus five pairs of strains from different patients were identical and one almost identical in phage pattern and serotype and a few with the same serotypes had minor differences in phage patterns. So far as conclusions can be drawn from these limited data, about one-third of the 20 patients who became colonized in the unit were cross-infected from other patients.

Patients, 4, 6, 14, 17 and 19 (Table 3) from each of whom another patient was thought to be cross-infected, had burnt areas of only 9, 7, 10, 8 and 5 % respectively. Only patient 26 among the six who might have been the source of cross-infection of another patient, had extensive burns (44 %). Patients 7, 16, 18, 20, 22, 23 and 30, with burns of 45, 65, 40, 65, 42, 65 and 40 % respectively, though needing much closer and more prolonged attention from nursing staff and, presumably, shedding far more pseudomonas, do not appear to have caused cross-infection of other patients. Patient 4 was sharing a double room with patient 5 who later became colonized with the same strain; patients 6, 14, 17 and 19 were all very elderly and almost immobile, they could not therefore have transmitted their strains by personal mobility in the unit. The apparent anomaly that cross-infection was not seen from patients with the heaviest colonizations of pseudomonas is considered in the discussion.

Analysis of strains from basin outflows

The common presence of *P. aeruginosa* in water traps below sinks, sluices and bath outflows in hospitals has long been believed to be a cross-infection hazard and an association between types in sinks and burns has been reported (Kohn, 1966). In the new burns unit there are 29 outflows from sinks and baths. These were not swabbed when the unit was first opened. In May 1968 after a year's working *P. aeruginosa* was isolated from 7 of them and in April 1969 after 2 year's working, from 11. For the next 3 months positive outflows were treated with a

caustic powder flushed down with boiling water, which sterilized them temporarily after each application. This experimental practice was stopped in August 1969 because on comparing types isolated from patients' burns with types isolated from sink units in their rooms it seemed clear that patients were seldom, if ever, infected from this source, an observation in agreement with that reported by Jellard & Churcher (1967) from a premature baby unit and by Lowbury *et al.* (1970) in a study on pseudomonas infection in patients with tracheostomy. Subsequently the number of infected outflows rapidly increased to 18 in October 1969, 24 in November 1969 and 27 in January 1970. These findings are shown in Fig. 1.

Table 4. *Serotypes of* Pseudomonas aeruginosa *recovered from basin outflows in relation to serotypes recovered from patients*

(New Unit, May 1968 to October 1969. UT = Untypable.)

	Outflows		Patients		
Serotype	Number of strains	Number of outflows	Imported	Developed in unit	Strain in room related to patient
1	13	8	0	0	—
4	3	3	1	0	—
9	6	6	0	0	—
10	21	15	0	0	—
13	1	1	0	0	—
UT	2	2	0	1	—
6	3	3	1	8	2
11	5	4	4	2	1
5C	1	1	1	1	1
5D	1	1	0	1	1

Up to October 1969, 56 strains grown from outflows were typed. Distribution according to serotype is shown in Table 4. In four of the five strains associated with patients colonized with the same type the outflow was probably infected from the patient. By far the greatest number of isolations were of serotypes 1, 9 and 10. Serotypes 1 and 9 were not isolated from any patient in this unit; serotype 10 was isolated once, imported on a patient in July 1968.

In November 1969 basins in six rooms were selected for more intensive study. From each positive primary culture a typical colony at the end of the spread representing the predominant strain was subcultured for typing and also any other colonies showing morphological differences. Table 5 shows the serotypes recovered from these outflows. The transient presence of serotype 6 in rooms B and E followed the residence of patients colonized with type 6 in each. The presence of serotype 11 for 2 months in room C followed the residence of a patient colonized with type 11 in that room. The transient presence of serotype 5D in the basin outflow in the treatment room D preceded colonization with type 5D on a patient who had been dressed in that treatment room. The rare serotype 13, not found on any patient, was recovered once in May 1969 from room C and once from room B 8 months later. Serotype 1, not found on any patient, and serotype 10, imported once on a patient, gained almost undisputed possession of this environment.

Colonizations with Gram-negative bacilli other than pseudomonas

These organisms consisted chiefly of strains of *Escherichia coli* and *Proteus* spp. *Klebsiella aerogenes*, though often isolated from other parts of the hospital, was not often found colonizing burns. In 1963–4, in 21 months in the old environment, burns on 39/208 (19 %) patients, of whom eight also harboured *P. aeruginosa*, were found colonized at some time. In 1967–9, in the first 30 months in the new environment, burns on 79/320 (25 %) patients, of whom 13 also harboured *P. aeruginosa*, were found colonized at some time.

Table 5. *Serotypes of* Pseudomonas aeruginosa *recovered from selected basin outflows*

Room	9. iv.	14. v.	28. ix.	10. ix.	7. xi.	13. xi.	17. xi.	27. xi.	7. i. 70
A	10	—	10	10	1	1, 10	1, 10	1, 10	1, 10
B	—	6	10	10	10	10	10	10	1, 13
C	6	13	—	11	11	10	11	10	10
D	5D	—	—	—	1, 10	—	1, 10	1, 10	—
E	10	UT	—	6	1, 10	1, 10	1, 10	10	1, 10
F	10	9	1	1	1	1	1	1	1

Table 6. *Numbers of patients annually with burns colonized by* Streptococcus pyogenes

	1963	1964	1965	1966	1967	1968	1969
Patients	128	161	147	137	170	169	160
Colonized with *Strep. pyogenes*	25	27	43	40	51	71	55
Percentage	20	17	29	29	30	42	34

Colonizations with Streptococcus pyogenes

The régime which is believed to have kept colonizations with *P. aeruginosa* at a reasonably low level for the past 7 years was much less successful in preventing colonizations with *Strep. pyogenes*. Table 6 shows, for each of the 7 years 1963–9, the numbers of patients from whom swabs were cultured and the numbers who harboured *Strep. pyogenes* in their burns at some time. The infection rate rose sharply in the new unit, one reason for which became apparent on observation of the behaviour of individual strains after importation.

Analysis of streptococcal types isolated from patients and the numbers of patients colonized with each, for the last 52 months in the old environment and the first 27 months in the new, is shown in Table 7. The comparison is not quite exact because typing records were available for only 65 % of the strains isolated in the old unit and for over 90 % in the new. However, it is clear, both from the variety of serotypes and from observing multiple introductions of the same type at different times, that importations of streptococci were very common in both environments.

Some types that were repeatedly imported did not spread, others were seen to spread only to a limited extent and a few caused epidemics of cross-infection. For the purpose of this study a strain was considered to cause an epidemic when, after

importation, it resulted in cross-infection of six or more patients. Eight such 'epidemics' are shown in Table 8, six of which occurred in the first 27 months in the new environment and only two in twice that length of time in the old. It is, however, plain from the 12 cross-infections caused by the strain of type 24 that epidemic spread could occur in the old environment as well as in the new. The most striking difference is in the 44 patients cross-infected by type 4 during three

Table 7. *Serotypes of* Streptococcus pyogenes *colonizing patients in the last 52 months in the old unit and the first 27 months in the new*

(TNF = Type not found. Figures in brackets = number of patients.)

M type	T pattern	Number of patients: Old unit	Number of patients: New unit	M type	T pattern	Number of patients: Old unit	Number of patients: New unit
1	1	1	7	2 (2)	2 (4)	4	—
Not tested	4	2	54	TNF	3	3	2
12	12	—	19	5 (2)	5/27 (3)	3	—
TNF	12	4	11	6 (1)	6 (3)	1	2
24	4/28	18	—	9 (1)	9 (3)	—	3
28 (R type)	28, 4/28	9	8	TNF	11, 3/11	8	—
TNF	28, 4/28	7	6	TNF	14	1	1
22 (1)	22 (9)	9	—	17	17/22/47	1	—
TNF	3/B3264	6	9	TNF	18	2	—
TNF	8/15/25/Imp 19 and variants	6	10	—	—	—	—

Table 8. *Epidemics of cross-infection with individual types of* Streptococcus pyogenes *following single importations*

(TNF = Type not found. NT = Not tested.)

M type	T pattern	Patients cross-infected: Old unit	Patients cross-infected: New unit
NT	4	—	25
12	12	—	14
24	4/28	12	—
NT	4	—	10
NT	4	—	9
1	1	—	7
28 (R)	4/28	—	7
TNF	22	6	—
	Totals	18	72

importations into the new unit; only two isolations of type 4 were recorded in the old. During the episodes of type 4 cross-infection, nasal and throat swabs taken repeatedly from the staff only once yielded a positive result, from a throat swab. On the other hand, the strain of type 1 which caused cross-infection of seven patients was imported by a member of the nursing staff with pharyngitis. It then caused pharyngitis in five other nurses who clearly could have infected patients before they could be removed from duty. Hence the apparently high communic-

ability of strains of type 4 in the new unit owed nothing to naso-pharyngeal infection among the staff and could account for most of the increased streptococcal colonization rate. The further possibility that conditions in the new environment somehow favoured streptococcal shedding and spread is considered in the discussion.

Colonizations with Staphylococcus aureus

Between 1961 and 1967, 357 strains of penicillinase-producing *Staph. aureus* from burn cultures were phage typed. Predominant phage patterns occurred in the chronological order 52/52A/80/81, 80/81, 77, 80/81, 85 and 52/52A/80/81. Other more heterogeneous Group III patterns were also prevalent in 1961–2 and 1965–7 and, probably, an untypable strain in 1964–5.

In 1963–4, in 21 months in the old environment, burns on 166/208 (80 %) patients were found colonized with *Staph. aureus* at some time; 100/166 (60 %) staphylococcus-colonized patients were shown to carry penicillinase-producers. In 1967–9 in the first 30 months in the new environment, 271/320 (85 %) patients were found colonized with *Staph. aureus*; 231/271 (85 %) staphylococcus-colonized patients were shown to carry penicillinase-producers. Thus, the proportion of patients colonized with penicillinase-producers rose from 60 to 85 %. In these two periods 42 (18 %) and 164 (36 %) patients were colonized with *Strep. pyogenes*. Those from whom *Strep. pyogenes* and penicillinase-producing staphylococci were at some time isolated from the same swab were 20 (10 % of patients) and 107 (33 % of patients).

DISCUSSION

The new environment did not reduce colonization rates of patients with *Strep. pyogenes*, *Staph. aureus*, *P. aeruginosa* or other Gram-negative bacilli. Indeed the streptococcal infection rates rose sharply though reasons have been given for believing that this was due to the presence of strains of increased communicability. Yet two features peculiar to the new unit may more than counterbalance the effect of the improved segregation of patients and protection in their rooms from airborne infection. The continuous movement of air in the new unit was observed clinically to effect more satisfactory surface drying; perhaps this resulted in increased streptococcal shedding from eschars. The continuous flow of air from above a patient's bed, perceptible to anyone who stands there, may be expected to carry infected particles from burn eschars directly against the persons of staff performing dressings and also into the passages. Transfer of such particles by staff was prevented as far as possible by changing gowns between one room and the next. However, some patients who had not left their rooms since admission became cross-infected with streptococci. During a wave of infection with *Strep. pyogenes* type 4, this organism was grown from swabs from floors, other surfaces, curtains and toys in a room occupied by a child shedding it, and also from stored skin from this child. In the passages outside such infected rooms an average of 0·6 airborne particles of *Strep. pyogenes*/cu.ft. was found by slit-sampling. It was also recovered on one occasion from the hair of three out of six nurses who had been in contact

with infected patients. Clearly, direct contact with staff was the most likely means whereby such patients became cross-infected with streptococci.

Staphylococci and streptococci are shed from colonized burns in far larger quantities than from any other source. Counts by the agar sausage method (Ten Cate, 1965) showed little difference between the old and the new units but equilibrium levels on various floor surfaces in both carried six to twenty times as many staphylococcal particles as corresponding surfaces in medical and surgical wards in the same hospital. Ayliffe *et al.* (1967) showed that fall-out bacteria on floors do not readily become redispersed but it is reasonable to assume that the staphylococcal equilibrium level on a floor is a fair measure of the numbers that have been shed and were once airborne. The increase in proportion of penicillinase-producing staphylococci from 60 % of patients in 1963–4 to 85 % of patients in 1967–9 may be the result of the continuous selective pressure maintained by treating streptococcal infections with penicillin, and the common presence of penicillinase-producing staphylococci with *Strep. pyogenes* may have prolonged surface carriage of streptococci and contributed to the rather high streptococcal colonization rates.

The high-rate of colonization with Gram-positive cocci contrasts with the low prevalence of *P. aeruginosa*. The observations of Govan & Gillies (1969) on pyocine typing of multiple single colonies from patients' lesions lend some support to the belief that all or nearly all of the pseudomonas colonizations listed in Table 3 were single strain infections, at least in the early stages. No less than nine different serotypes (and within serotypes numerous differences in phage pattern) were seen among the strains from the 30 patients shown to be colonized with pseudomonas in the first 32 months in the new unit, showing the frequency of importation of unrelated strains. Although the survival time for pseudomonas in air is short Barclay & Dexter (1968) found in another new burns centre that when the infection rate was high settle plates showed contamination with pseudomonas at heights all the way up to the ceiling. In this unit direct airborne spread from one room to another can be discounted. Yet even though the general degree of colonization was low, about one-third of the 20 patients developing colonizations in the unit appear to have been infected from another patient.

The origin of the infections of the other two-thirds of the 20 patients who were colonized with strains of pseudomonas not previously seen in the unit or apparently not present at that time is more debatable. No patients became colonized from the many basin outflows heavily infected with serotypes 1 and 10 and patient-colonizations with pseudomonas were at a minimum during the last 4 months of 1969 when nearly every outflow became contaminated with it and also during the first 5 months of 1970. On five occasions the strain from a colonized patient was recovered transiently from the basin outflow in the corresponding room, but only in one instance did the timing of isolations indicate that the outflow was colonized before the patient.

Had it been practicable to type multiple single colony subcultures of serial swabs from patients colonized with pseudomonas, mixed infections with minority populations might have been revealed; and had swabs been cultured from fluid medium, scanty growths would probably have been shown in some patients

recorded as free from pseudomonas. However, such minor sources are quantitatively negligible compared with the massive shedding from patients with high percentage burns colonized with identified strains of pseudomonas, from whom cross-infection was not observed. Lowbury & Fox (1954) showed that the hands of nurses in a burns unit were often contaminated with pseudomonas and Lowbury *et al.* (1970) showed that strains of pseudomonas infecting patients with tracheostomy were often isolated from the hands of nurses, physiotherapists and other staff. Hand swabs were not taken in this study but pseudomonas from heavily infected patients was likely to be present on hands of staff from time to time. Streptococcal and staphylococcal cross-infection was certainly transmitted by staff; why, therefore, was cross-infection with pseudomonas not seen from the very patients most heavily colonized?

The four North Africans, each nursed and dressed in a separate room from which he did not emerge for a fortnight, were all shown to become rapidly cross-infected with penicillinase-producing staphylococci and also with *Strep. pyogenes* type 12, the prevalent strain at that time. Three also became colonized with pseudomonas, each with a different type, on the 3rd, 14th and 16th days at a time when no other patient was colonized with pseudomonas and none of these three types were grown from other environmental sources. Perhaps these patients had no previous experience of such organisms and were specially host-receptive to minimal contamination from undisclosed sources. Pseudomonas appeared first on chest, back and neck respectively, not on 'pyoprone' (i.e. bathing-trunk) areas (Sachs & Watson, 1969), a point against self-infection from the bowel. This, however, remains a possibility and in one series, *P. aeruginosa* was found in the faeces of nearly a quarter of patients on admission to hospital (Shooter *et al.* 1966).

Of the other patients, two were colonized on fresh grafting sites, one was colonized with a strain previously found in the sink outflow in the treatment room where he was dressed, three had been treated in other hospitals for more than a week and had moist eschars on admission and one had blisters stripped elsewhere before admission. The balance of evidence, therefore, suggests cross-infection as the most common source of colonization with strong differences in host receptiveness to account for the capricious absence of observed cross-infection from the most heavily colonized patients.

The many contaminations in basin outflows with strains of serotypes 1 and 10 suggest multiple infections from a common source. Recovery of serotype 9 from six basin outflows on one occasion only and of serotype 4 from three basin outflows on one occasion only, also suggests contamination from a common source. In these basins hollow tubed plugs replace overflow ducts and metal bulbs below right-angled bends replace U-traps. Each waste pipe then passes vertically through the floor into the top of a sloping 4-in. metal drain pipe, bacterial contamination from which is very unlikely. Water in the outflow traps remains uninfected so long as the rooms remain unused. In occupied rooms bacterially contaminated material regularly disposed of in basins included tooth water, washing water from non-burnt parts of patients' bodies and water from cloths (individual to each room) after wiping down surfaces. A separate floor mop was used for each room and the

cleaning staff had orders not to raise mop heads above patients' floor surfaces. Pseudomonas was sought in floor mops, room cloths, flannels, toilet bowls, liquid soap dispensers, peppermint water, water taps and water samples. Strains of serotypes, 2, 3, 10 and 11 but not 1 were recovered from mop heads but the reason for the abundance of types 1 and 10 in outflows remains obscure unless the environment specially favoured colonization from minimal unobserved contaminations with these types.

I thank Mr J. Laing, Director, Mr D. C. McNeill, Sister J. Harvey, Sister J. Cotterell and other members of the staff of the Wessex Regional Burns Unit for unstinted help and facilities over many years, Dr M. T. Parker and Mrs D. Martin for typing strains of pseudomonas, Dr W. H. H. Jebb and Mr F. C. Saunders for typing strains of streptococci, Dr G. Thomas and Mr C. M. R. Pitman for undertaking slit-sampling and Mr C. Dowding for much technical help. I am also grateful to Dr E. J. L. Lowbury and to Dr M. T. Parker for valuable discussions.

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J. Hyg., Camb. (1970), **68**, 647
Printed in Great Britain

Viruses associated with acute respiratory infections in Royal Air Force personnel

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(*Received* 13 *July* 1970)

SUMMARY

All respiratory illnesses which were reported to the medical officers between September 1966 and December 1967 on a Royal Air Force station of 350 men were studied virologically.

Three periods of increased respiratory infections were observed: two occurred in the autumn, one in each year, and the third in the winter during January and February. The autumnal outbreaks were associated mainly with rhinovirus infections, and high isolation rates (82·1, 65·9 %) were achieved at these times. Few of the illnesses during the winter outbreak could be diagnosed in the laboratory, and no evidence was found of infection with 'coronaviruses'.

Despite the entrance of 30 fresh recruits direct from civilian life every 5 weeks, the respiratory infections encountered on the station were very similar to those in the local population and were not predominantly infections with adenoviruses, Coevirus, and *Mycoplasma pneumoniae*, as previously reported from larger military recruit centres.

INTRODUCTION

Many workers have reported studies of the acute respiratory infections experienced by new entrants to the armed forces, most of them at large initial training establishments. The value of many such investigations, however, has been limited by the small proportion of cases in which a diagnosis could be confirmed in the laboratory.

In 1967 it became possible for us to investigate the respiratory illnesses in a Royal Air Force station which received small intakes of officer cadets straight from civilian life to undergo their initial aircrew training. The purpose of the study was to compare the importance of the viruses isolated from respiratory illnesses in this small unit with those reported from larger recruit centres. Furthermore, it was hoped that a combination of serology, a range of tissue cultures, and the use of organ cultures would allow a diagnosis to be made with sufficient frequency that the major causes of respiratory infections could be defined.

MATERIALS AND METHODS

Population studied

All Royal Air Force personnel with acute respiratory illness who reported to the Station medical officers at R.A.F. South Cerney between September 1966 and December 1967 were included in this study. The total complement of the station was approximately 350 officers and men and its main function was the initial training of officer cadets for aircrew duties. Cadets were admitted direct from civilian life to a course lasting 15 weeks. Each course consisted of approximately 33 cadets and every 5 weeks one course graduated and another course began its training so that three courses (*ca.* 100 cadets) were present on the station at any given time.

Specimens

A nose swab, two throat swabs, and a specimen of blood were taken from each patient at the initial interview and a second specimen of blood was drawn between 2 and 3 weeks later, if the patient had not been posted by this time.

Virology

One throat swab was transported dry to the laboratory and inoculated on a blood agar plate. The nose swab and second throat swab in transport medium were held and sent to the laboratory at 4° C. All such specimens were examined in tissue cultures of monkey kidney, the Bristol line of HeLa cells, human embryo diploid fibroblast (WI-38), and human embryo kidney (HEK), and newborn mice were inoculated—all as previously described (Higgins, Ellis & Boston, 1963; Higgins, Boston & Ellis, 1964). Viruses isolated were identified by haemadsorption inhibition, haemagglutination inhibition, or neutralization test except for rhinoviruses which were confirmed by their ability to grow in rolled cultures at 33° C. at pH 7 but not in stationary cultures with alkaline medium at 37° C. or by their instability in acid medium.

Specimens which failed to yield an agent by these methods were inoculated into organ cultures of human embryonic ciliated epithelium (Hoorn, 1966), the fluids from which were tested in tissue cultures and newborn mice as described for the original specimens. No fewer than four passes in organ culture, the last of which, at least, was by the modified method of Tyrrell & Blamire (1967), were carried out on all specimens which failed to yield a virus. The fluid from the final pass was spun at 60,000**g** for 30 min., the pellet resuspended in a few drops of distilled water and applied to a formvar or formvar–carbon-coated grid and stained with 2% phosphotungstic acid at pH 6·9. The grids were then examined in a Hitachi HS 7S, a Phillips 100, or a Phillips 200 electron microscope.

Serology

Paired sera were tested by the method of Bradstreet & Taylor (1962) for fourfold or greater rises in complement-fixing antibodies to influenza A, B and C viruses, psittacosis, Q fever, adenoviruses, respiratory syncytial virus, Sendai virus, and *Mycoplasma pneumoniae*. Sera from cases which failed to yield an agent were also tested for antibodies against the 229E (Hamre & Procknow, 1966) strain of

'coronavirus' (Almeida *et al.* 1968) by the same method and against the OC-43 strain of 'coronavirus' (McIntosh, Becker & Chanock, 1967) in a similar fashion but using $1\frac{3}{4}$ units of complement.

The 229E antigen was an infected WI-38 tissue culture fluid and the OC-43 antigen an infected mouse brain suspension. All other antigens were supplied by the Standards Laboratory, Colindale.

RESULTS

Three periods of increased respiratory illness were observed during the period studied (Fig. 1). Between September and December 1966, 39 cases of acute respiratory infection were seen; 35 were seen between January and March 1967; and 44 between September and December 1967, an incidence of 7·96, 8·33 and 7·49/1000 persons/week respectively. From April to August 1967 only 11 patients with respiratory infections consulted the medical officers, an incidence of 1·43/1000/ week.

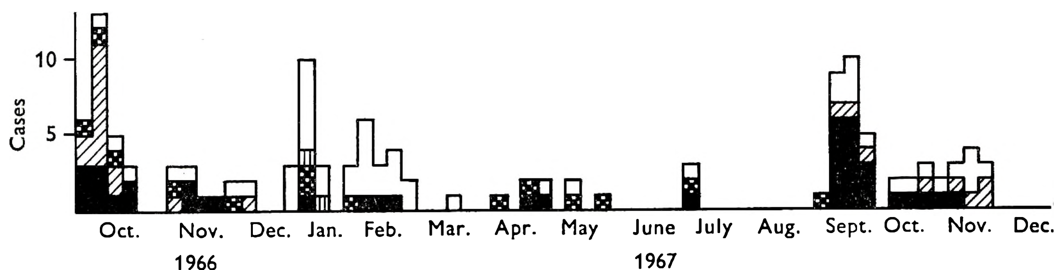

Fig. 1. Virological diagnosis of cases of acute respiratory infections seen between September 1966 and December 1967. ▨, Rhinovirus M; ■, Rhinovirus H; ▩, other pathogens; ▥, diagnostic serology; □, illness without isolation of pathogens or diagnostic serology.

Isolations

A total of 77 agents (Table 1) were isolated from 76 (59 %) of the 129 illnesses. Of the infecting agents, 62 (80·5 %) were rhinoviruses with H-types isolated twice as frequently as M-types. A quarter of the H-type rhinoviruses were isolated only in organ culture and of the 30 isolations in tissue culture 22 were detected in HEK cultures but only 17 in WI-38. Similarly, seven of the 22 M-type rhinoviruses were isolated only in organ culture and a further six only in monkey kidney cultures. Eight strains were detected in both monkey kidney and WI-38 and one in monkey kidney, WI-38 and HEK. The strains isolated only in monkey kidney were readily isolated from the specimens but proved increasingly difficult to pass or adapt to other tissue cultures so that typing of these strains was not possible. Typing was attempted with 11 other rhinoviruses, one of which was not identifiable with the 22 sera available. Of the ten strains which were typed, three belonged to type 4, two to each of types 1 B and 29, and one to each of types 1 A, 15 and 30.

No structures resembling avian infectious bronchitis virus or the parainfluenza group of viruses were seen on electron microscopy of the concentrated fourth or later passage organ culture fluids of the specimens which failed to yield an agent detectable in the tissue culture systems employed.

Distribution of isolations

The most striking feature of this survey is the comparison between the high isolation rates associated with the two autumnal outbreaks of respiratory illness and the quiescent period during the summer, 82·1, 65·9 and 72·7 % respectively, and the failure to detect an infecting agent for the majority of illnesses in the early part of 1967, when the isolation rate was only 20 % (Fig. 1).

Table 1. 77 *Isolations from* 76 *of* 129 *respiratory illnesses between September* 1966 *and December* 1967

No. of strains	Agent
40	Rhinovirus H-type
22	Rhinovirus M-type
4	Herpes simplex virus
2	Poliovirus type 1
1	Parainfluenza virus type 1
1	Coxsackie virus type A10
1	Coxsackie virus type B3
1	Adenovirus type 5
1	β-haemolytic streptococci Group A*
4	β-haemolytic streptococci Group G
77	

* Rhinovirus H-type also isolated.

Serology

Paired sera were examined from 49 of the 76 patients who yielded an agent, but no rises in antibody were demonstrated. This was because the agents isolated were mainly rhinoviruses and other viruses, antigens to which were not used in the complement fixation test. A rise in antibody titre to respiratory syncytial virus antigen from 1 in 16 to 1 in 64 in one pair of sera and against both adenovirus and influenza C virus antigen from 1 in 8 to 1 in 32 in another were the only positive findings among the 42 paired sera from patients from whom no virus was grown. No rising titre to either 'coronavirus' was detectable and no serum contained antibody at 1 in 8 to 229E but five pairs of sera had unchanging titres between 1 in 8 and 1 in 32 to OC-43 antigen.

Association of virus with clinical illness

The frequency with which each clinical type of respiratory illness was seen and the agents isolated from these cases is shown in Table 2. Sore throats accounted for half the respiratory infections seen; colds and tracheitis were each responsible for approximately a quarter of the cases. One case of laryngitis was the only other infective respiratory condition diagnosed during the period. Rhinoviruses were isolated from almost two-thirds of patients suffering from colds or tracheitis but from little more than one-third of those with sore throats. All isolations of enteroviruses and herpes simplex virus and of four of the five streptococci were associated with sore throats.

The severity of the illness may be measured by whether the patient was suffi-

ciently ill to warrant admission to sick quarters or could be treated as an out-patient, and also by the length of time the patient was off duty. This information was available for 77 of the cases seen during the first year and its relationship to the agents isolated is summarized in Table 3. The number of infections with viruses other than rhinoviruses and those with streptococci are too small to comment on with the exception that all four streptococcal infections were considered to need in-patient treatment and that the average time off duty for all patients with 'other virus' infections was of the same order as that for in-patients from whom no agent or a rhinovirus was isolated. There is a close similarity between the proportion of patients admitted and those treated as out-patients, the range and mean duration of time off duty for rhinovirus infections, and for those where no agent was isolated.

Table 2. *Laboratory diagnosis in relation to clinical illness*

Diagnosis	Cold	Pharyngitis	Tonsillitis	Tracheitis	Laryngitis
Agents isolated	1 Parainfluenza virus type 1	8 Rhinoviruses type H	8 Rhinoviruses type H	13 Rhinoviruses type H	Nil
	11 Rhinoviruses type H	8 Rhinoviruses type M	3 Herpes simplex viruses	6 Rhinoviruses type M	—
					—
	8 Rhinoviruses type M	1 Herpes simplex virus	1 Coxsackie virus type A10	1 Adenovirus type 5	—
	1 Streptococcus group A†	2 Poliovirus type 1	3 Streptococcus group G	—	—
	—	1 Coxsackie virus type B3	—	—	—
	—	1 Streptococcus group G	—	—	—
Fourfold rise in antibody	Nil	1 Respiratory syncytial virus	Nil	Nil	Nil
	—	1 Influenza C virus*	—	—	—
	—	1 Adenovirus*	—	—	—
Negative	12	11	16	11	1
Total	32	34	31	31	1

* Same pair of sera. † Rhinovirus H-type also isolated.

Table 3. *Severity of illness in relation to laboratory diagnosis*

Isolate	Rhinoviruses		Other viruses		Streptococci		No agent	
Admitted to sick quarters	Yes	No	Yes	No	Yes	No	Yes	No
Number of patients	20	12	6	2	4	0	23	10
Days off duty: Range	2–5	1–2	2–3	2–3	2–4	—	2–6	1–2
Total	51	23	15	5	11	—	59	19
Days off duty: Mean	2·55	1·92	2·50	2·50	2·75	—	2·57	1·90

DISCUSSION

The view that a comprehensive range of tissue cultures and the use of organ cultures would improve the results in the study of acute respiratory infections is justified. The limitations of the use of only HEK or WI-38 for the isolation of rhinoviruses (Higgins, 1966*a*) are confirmed, as are the advantages of employing organ cultures of ciliated epithelium when attempting to detect these viruses (Tyrrell & Bynoe, 1966; Higgins, 1966*b*).

The agents most commonly isolated were not adenoviruses, Coxsackie virus type A 21, and *Mycoplasma pneumoniae*, as previously reported from larger military establishments (Johnson, Bloom, Mufson & Chanock, 1962; Chanock, Fox & James, 1967; Oei & van der Veen, 1967; Mantyjarvi *et al.* 1967; Mogabgab, 1968; van der Veen, Oei & Abarbanel, 1969) and the preponderance of rhinovirus infections more closely resembles that found in the local population (Higgins, 1967) or among university students (e.g. Gwaltney & Jordan, 1966; Hamre, Connelly & Procknow, 1966; Phillips, Melnick & Grim, 1968) and families (e.g. Hendley, Gwaltney & Jordan, 1969; Fawzy *et al.* 1967). These findings would indicate that a unit of 350 men with intakes of 30 fresh individuals at regular intervals is too small for the introduction of many new viruses or possesses too few susceptibles to support an outbreak. Although the serotyping of rhinoviruses was very limited, at least seven different serotypes were detected among the 11 strains tested showing that no period of increased respiratory illnesses was attributable to an outbreak with one particular serotype.

The failure to determine the cause of the majority of the respiratory infections in the early part of 1967 by the use of tissue culture had led us to believe that the illnesses were likely to be the result of infection with 'coronaviruses'. This may be so although we failed to demonstrate the presence of typical virus particles in fluid from the organ cultures infected with these specimens or to show a rise in antibody to 229E or OC-43 in paired sera from these cases. This work was begun before Tyrrell & Blamire (1967) reported the importance of the modified method of organ culture for the isolation of these viruses; and although the later organ culture passages were by this method the earlier ones were performed as originally described by Hoorn (1966) and this could have prevented the growth of these viruses. Furthermore, strains of 229E virus have been isolated in diploid fibroblast of human embryonic intestine (Kapikian *et al.* 1969) but the authors failed to grow the strain in organ cultures of ciliated epithelium and this may apply to other 'coronaviruses'. Not all 'coronaviruses' so far isolated are serologically related to either 229E or OC-43 (McIntosh *et al.* 1969) so that negative serological findings do not exclude the possibility that these infections were caused by 'coronaviruses'. However, the close similarity in severity and duration of the illnesses from which no agent was isolated and those where a rhinovirus infection was demonstrated suggests that the causal organism was more likely to be a fastidious rhinovirus which cannot be detected in tissue cultures similar to HS (Hoorn & Tyrrell, 1966) and ST (Tyrrell, Bynoe & Hoorn, 1968) for the illness associated with 'coronavirus' infections has been shown to be shorter than those following infection with rhinoviruses (Bradburne, Bynoe & Tyrrell, 1967).

We are indebted to Mr E. O. Caul of the Public Health Laboratory at Bristol for the electron microscopy of a number of the organ culture fluids and to Dr A. H. T. Robb Smith, Director of Pathology, Radcliffe Infirmary, Oxford, for facilities to examine the remainder. We also acknowledge the continuing help of Dr H. E. M. Kay of the Royal Marsden Hospital and the gynaecologists and staff at the Victoria Hospital, Swindon, and St Paul's Hospital, Cheltenham, in supplying foetal material. We are grateful to the Director-General of Medical Services, Royal Air Force, for permission to publish this work, which was performed with the aid of a grant from the Medical Research Council.

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J. Hyg., Camb. (1970), 68, 655
Printed in Great Britain

Respiratory disease in a colony of rats

I. The natural disease

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SUMMARY

An epidemic of acute respiratory disease in a colony of CFE rats is described, the main laboratory findings are recorded and its aetiology discussed.

The epidemic showed that severe respiratory disease varying from peracute to chronic was associated with infection of the lungs with a mycoplasma but that mycoplasmas could be present in rats, even in the lungs, without signs of disease, thus suggesting that one or more other factors were involved. It is also evident that there are strain differences in the susceptibility of rats to this disease.

INTRODUCTION

A breeding colony of approximately 300 gnotobiotic CFE strain rats was established behind a barrier against infection in newly built quarters at Alconbury, England, in June 1966. The foundation stock was from germ-free animals that had received an enteric flora, consisting of a group N Streptococcus, a Lactobacillus and a Bacteroides.

By May 1968 eight additional micro-organisms had been identified in the colony, namely *Staphylococcus albus*, *Bacillus subtilis*, *Clostridium welchii*, *Escherichia coli*, *Bacterium aerogenes*, *Proteus* spp., *Penicillium* spp. and *Aspergillus* spp. There was no evidence of ectoparasites, helminths, protozoa, pasteurella, pseudomonas, mycoplasmas or viruses.

The general health of the colony was excellent until the summer of 1968 when an acute respiratory disease broke out; by September it had reached epidemic proportions. In this paper the disease and its attempted control are described and the possible aetiology discussed.

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MATERIALS AND METHODS

Species and strains

CFE rats. In June 1966 the foundation stock was obtained from a colony at Carworth, U.S.A., which had been derived originally from the Sprague–Dawley strain and maintained as an outbred closed colony for some 25 years.

CFHB rats. From May to July 1968 a strain of Wistar origin was introduced by the 'dry' hysterectomy method.

CFY rats. From July 1968 to January 1969 a third strain was introduced by the 'wet' hysterectomy method. This strain was derived from rats obtained from the Charles River Breeding Laboratories, France.

CFLP mice. From April to August 1967 a colony of ICI Alderley Park strain 1 mice was established by the 'dry' hysterectomy method. The pups were initially fostered onto gnotobiotic mice of a different strain previously introduced into the 'red area'.

Environment

Each strain was housed separately as far as possible within the area behind the barrier, which was called the 'red area', and comprised some 5000 ft.^{2} (465 m.^{2}) of animal rooms and 2500 ft.^{2} (232 m.^{2}) of corridors, stores and other service areas.

Incoming air was filtered to exclude particles larger than 5 μ, heated to about 74° F. (23° C.) and humidified to about 60 % R.H. Room air was changed 12 times/hr.

All materials entering the 'red area' were subjected to a decontamination process. The food was autoclaved at 105° C. for 10 min.; cages, general hardware and clothing at 120° C. for 10 min.; cage tops at 130° C. for 3 min.; bedding at 135° C. for 10 min. Other materials were passed through a 'dunk' tank containing a disinfectant,* or were surface-sterilized by formalin in a gas lock.

Staff entering the 'red area' took a shower and changed into autoclaved clothes. No masks were worn. The hands and forearms were rinsed with 70 % alcohol on entry and several times a day. Entry was prohibited to anyone having contact elsewhere with rats or mice during the previous 3 weeks; only very rarely were visitors allowed in.

Husbandry

The animals were housed in polypropylene cages which had galvanized iron tops and which stood on open shelves on metal racks. Sawdust and wood shavings with as low as possible a content of fine dust were used for bedding, and paper for nesting.

The feed was a high fat high protein diet.† Drinking water was acidified (pH 2·0–2·5) with hydrochloric acid. Breeding methods were intensive, but avoided closely related mating. Growth and reproduction rates were high.

* Task: British Hydrological Corporation, Wimbledon.
† Carworth–Dixon diet. E. Dixon and Sons Ltd., Ware.

Hysterectomy

The CFHB rats, the CFY rats and the CFLP mice were all introduced into the 'red area' by hysterectomy and fostering. The donor dam, judged to be within 24 hr. of full term, was killed by hyperextension of the neck and cervical fracture, immersed in warm freshly prepared 1% iodophore solution, pinned out on the operating board, and, under frequent drenching with the iodophore solution, the abdomen was opened by a long median incision. The *cervix uteri* was ligatured, cut immediately caudal to the ligature and the uterus was freed, placed in a screw-capped jar of iodophore solution and passed through the dunk tank into the 'red area'.

It was found to be important that the temperature of the iodophore solution should be 39–40 °C.

The donor dam's thorax was opened immediately after the removal of the uterus and before this was passed into the 'red area'. If there were macroscopic lesions on the lungs of the donor dam the uterus was rejected. In fact this happened on one occasion only.

When the jar containing the uterus in warm iodophore solution was received in the 'red area' one of two methods, called respectively 'dry' and 'wet', was employed. In the 'dry' method the uterus was removed from the iodophore solution, placed in a small dish and opened with scissors. The young with their attached placentas were removed and placed on a warm surface covered with a sterile paper towel. The pups were dried off and if necessary stimulated to breathe, and the placentas were removed. When the pups were bright pink in colour and moving actively they were given to the foster dam, which had had a litter 24–72hr. previously inside the 'red area'. Before fostering she had been removed from her cage, and her own pups taken away and subsequently killed with chloroform. The fosterling pups were placed in the nest with minimal disturbance. The dam was then returned to the cage and in the vast majority of cases adopted her fosterlings without trouble.

The 'wet' hysterectomy method differed from the above as follows: The uterus was opened up below the surface of the iodophore solution so that the pups were delivered in a bath of disinfectant; in this they were well rinsed before being removed from the solution and placed on the warm surface. Thus the maternal uterine tissue and its secretions never left the disinfectant solution and never came in contact with the air of the 'red area'.

Laboratory examinations

Histopathology

Specimens were preserved in 10% buffered formalin and were referred to Dr L. Mawdesley-Thomas, the Department of Pathology, Huntingdon Research Centre, Alconbury, Huntingdon.

Bacteriology

Most of the media were prepared by the methods of Cruickshank (1965). In the later part of this work liquid and solid media for the cultivation of mycoplasmas

were supplied by Dr P. Whittlestone, School of Veterinary Medicine, Cambridge. Their preparation is described elsewhere (Whittlestone, Lemcke & Olds, 1971).

Cultures were incubated at 37° C. and examined daily for at least a week. To reduce dehydration solid media were incubated in a plastic bag which contained a piece of moist paper. Liquid media were used in screw-capped bottles.

History of the epidemic

For 2 years the general health of the CFE colony was excellent, although there were sporadic spontaneous haemorrhages especially in heavily pregnant females during the latter part of 1967. There was also some evidence of marginal hypovitaminosis E. These conditions were corrected by increasing the content of vitamins K and E in the diet.

In January 1967 there were no macroscopic lesions in the lungs of these rats and histological examination showed only minimal amounts of lymphoid tissue, chiefly in the bronchiolar bifurcations. By the end of 1967 the amount of lymphoid tissue had increased considerably although there were no frank lesions and no clinical or post-mortem signs of respiratory disease. In January 1968 forty CFE rats were sent from Alconbury to Carworth U.S.A. for a general microbial screen; no evidence was found of respiratory disease or of infection with mycoplasmas or viruses.

In June 1968 an acute respiratory syndrome appeared in CFE females being used as foster mothers for pups of the CFHB strain. The clinical signs were hunched posture, a dull staring coat, severe respiratory distress and readily audible râles. The animals lost weight, rapidly became moribund and were removed from the colony.

Shortly afterwards other CFE rats in this room became similarly affected. After several weeks cases began to occur in the other rooms and very quickly the incidence in these rooms rose. By September 1968 the epidemic was widespread throughout the whole CFE colony. As the epidemic developed clinical disease was seen in progressively younger animals until at the height of the epidemic (November–December 1968) rats as young as 10 days showed respiratory distress and died. Extensive areas of bronchopneumonia were found in their lungs on post-mortem examination.

Chemotherapy of the epidemic

At its peak the disease was so severe that the whole colony seemed in danger of extinction. An attempt was therefore made to control the outbreak by using 1 % oxytetracycline hydrochloride in the drinking water. Although the immediate results were encouraging, after several weeks treatment it was apparent that the disease was only being contained. This drug was therefore discontinued and 0·6 % tylosin in the drinking water was tried instead. After some weeks of treatment preweaning mortalities fell to the pre-epidemic level; there was little evidence of pulmonary disease in adult rats, although some had otitis media from which mycoplasmas were cultured.

By March 1969 use of antibiotics had been substantially reduced. Mild clinical cases occurred and were being culled, but only occasional cases with severe respiratory involvement were seen.

RESULTS

Necropsy findings

On examination post-mortem some animals showed areas of red or grey hepatization in one or more lobes of the lung. Sometimes the pneumonic area consisted of discrete or confluent grey nodules often involving large areas of lung tissue. Frequently the trachea contained abundant mucus. Histologically there was evidence of an acute inflammatory reaction similar to that described by Mawdesley-Thomas (1968).

Most animals examined post-mortem had pus in the middle or inner ears. Sometimes the middle ear was so affected that the tympanic membrane was distended into the external auditory canal by yellow pus. More commonly, at necropsy no abnormality was found in the middle ear, but there was thick pus in the cavity of one or both cochleas, the lining membrane of which was thickened. Previously the animals so affected had no clinical signs of disturbance of the vestibular postural mechanisms. Except for a variable amount of mucus, no abnormality was found in the nasal cavity.

Virological examination

Three rats examined by Dr R. D. Barry, Department of Pathology, Cambridge had clinical signs of acute respiratory disease. At necropsy the lungs of one appeared normal; the other two had some pulmonary consolidations.

Three tenfold dilutions of homogenate of each affected lung were inoculated into allantoic or amniotic cavities of chick eggs and into primary cultures of monkey kidney cells. Dr Barry reported that there was no evidence for the presence of Sendai virus or of any other virus detectable by the methods used. These included haemagglutination tests on allantoic and amniotic fluids, and haemadsorption tests and examination for cytopathic effect in monkey kidney cells; all tests were made 72 hr. after inoculation. Culture fluids from monkey kidney cells were passaged to fresh cultures which were similarly examined also.

Bacteriological examination

Two advanced cases were killed with coal gas and examined for a possible bacterial cause. Their lungs showed typically affected areas, smears from which contained numerous polymorphonuclear neutrophils, but no recognizable bacteria. Tiny rods and cocci were found within neutrophils in Giemsa-stained smears.

The pneumonic areas of each lung were homogenized, one in saline and the other in peptone water. The following media were sown with each homogenate: for anaerobic incubation, Loeffler's serum, blood agar, heated blood agar and Albimi broth; for incubation in air + 5 % CO_2, the same media as well as serum penicillin agar and fluid thioglycollate medium. Eight mice (not from the CFLP

colony) were injected, some intraperitoneally and some subcutaneously, with 0·6 ml. of lung homogenate each.

After 6 days of incubation the blood agar plates showed tiny colonies surrounded by greyish zones of incomplete haemolysis. Amorphous films of growth were noticed on Loeffler's serum slopes. These were judged probably to be mycoplasmas, from the media on which they grew and the time of their appearance, from their minute colonies on enriched media, from their appearance by dark field microscopy, and from their failure to stain as recognizable bacteria by Gram's method. No eubacteria were recognized on any of the culture media.

Two of the three mice injected subcutaneously developed local swellings over the following 2–4 weeks. One was killed and the swelling was found to consist of an abcess containing semi-fluid pus composed virtually entirely of neutrophil polymorphs. A mycoplasma was grown in pure culture from the pus. The other affected mouse was observed for 3 months. The swelling was at its greatest size ($10 \times 10 \times 3$ mm) at 2 months, but had disappeared by 3 months. The third mouse which received a subcutaneous injection developed no lesion. It had been injected with the homogenate which contained the fewest organisms.

Five other mice injected intraperitoneally showed no clinical abnormalities and no lesions were found when they were killed and examined 15 weeks after injection. Subsequently 51 CFE strain rats from the colony were examined and cultured. Where typical pulmonary lesions were found mycoplasmas were consistently isolated. On the other hand mycoplasmas were sometimes grown from sites which showed no macroscopic evidence of disease, but usually lighter growth was found in these cultures.

Two clinically normal CFY strain rats from the colony were examined for the presence of mycoplasmas. No macroscopic lesions were found post-mortem in either of the lungs or the internal ears, but mycoplasmas were grown from the lungs and the ears of both rats.

DISCUSSION

These results suggested that the mycoplasma might be a cause of the disease, but that other factors were involved in producing the lesions.

The disease in the colony at Carworth Europe was first noticed after the introduction of hysterectomy-derived stock. The possible congenital transmission of the organisms is consistent with the findings of Graham (1963), who isolated *Mycoplasma pulmonis* from the ovaries or uterus of 23 of 77 female rats and produced genital lesions in female mice following intraperitoneal injection of cultures. He suggested that caesarean derivation might not eliminate mycoplasmas from the young of mothers with genital infection.

The genital tracts of three old female CFE strain breeders were examined and from the uterus of one pus was expressed which yielded abundant growth of a mycoplasma. On another occasion pus was found in the fallopian tube of a CFE rat from Carworth Europe which had been kept for some weeks in another laboratory. This too yielded a mycoplasma and Dr R. Lemcke confirmed that this was

serologically identical with that obtained from the respiratory tracts of the CFE colony in the course of the epidemic.

Although not enough animals have been examined to assess the frequency of genital carriage, it is clear that the mycoplasma isolated in this epidemic may be found in the female genital tract. This suggests that the dry hysterectomy method should not be relied upon to exclude mycoplasma from a barrier protected area, since contaminated uterine secretions would be exposed within the clean area. One might hope that the wet hysterectomy method would eliminate this hazard.

Mawdesley-Thomas (1968) brought forward histological evidence of an outbreak of acute bronchopneumonia in CFE strain rats which had been housed in the same animal rooms as clinically normal rats of the same origin as the CFY strain.

In May 1969 12 rats were obtained from the same source as that referred to by Mawdesley-Thomas, and were examined for the presence of mycoplasma immediately on arrival. They came by air and road in filter boxes and never had any contact with the Carworth Europe colony or rats coming from that colony.

At necropsy they showed no obvious clinical abnormalities, and no lesions that could be attributed to mycoplasma infection were found in the lungs, but in each of two females yellow pus was found in the middle ear. Mycoplasmas were grown from all 12 of these rats, from the inner ears of 11 and from the lungs of three. By serological and cultural tests the mycoplasma grown from these rats was identical with that grown from Carworth Europe rats (Whittlestone *et al.* 1971).

The disease in the CFE rat colony at Alconbury was clinically of an acute, even peracute, nature, and the histological picture was identical with that described by Mawdesley-Thomas (1968). Previously, mycoplasma-induced respiratory disease in rats has been referred to as 'chronic respiratory disease' (CRD). The relationship of mycoplasma to respiratory disease in rats, whether acute or chronic, has been discussed by Bell & Elmes (1969) and by Lane-Petter (1970).

Whatever the relationship between this organism and the disease it would appear to be preferable to discontinue using the phrase 'chronic respiratory disease' when referring to the syndrome, and to call it 'murine respiratory complex'.

Apparently healthy rats can carry mycoplasmas, particularly in their inner ears, without clinical signs of respiratory disease or macroscopic lung lesions.

Nelson (1963) associated the CRD syndrome with two agents, *M. pulmonis* and a virus. Tyrrell & Coid (1970) have reported acute respiratory disease in rats caused by Sendai virus. At the time of the outbreak of the disease in the CFE colony raised antibody titres to Sendai virus had been detected, and clinical disease attributable to this virus had been seen in the CFLP mouse colony housed in the same building. The possibility of its presence in the rats cannot be ruled out.

Gases such as sulphur dioxide, carbon monoxide and ammonia (Dalhamn & Reid, 1965) can produce marked changes in the lungs of rats. At the time of the outbreak a fault in the ventilation plant had allowed fumes, probably containing sulphur dioxide and carbon monoxide, to enter the 'red area' from the heating system. There was an appreciable ammoniacal smell in the animal rooms as the

result of a high stocking density of animals. These irritant gases could have played a part in lowering the resistance of the host to the mycoplasma. At the time of the outbreak sawdust and wood shavings were being used as bedding material and these produce dust, sometimes in considerable quantities.

It is possible that the severity of the disease was the result of the rat colony first being exposed to the mycoplasma at a time when the presence of other factors had lowered the host's resistance generally and in particular the resistance of the lungs. There does, however, seem to be a special strain susceptibility to this mycoplasma in CFE rats. Although the organism was found in the respiratory tracts of all three strains of rat, only in the CFE strain did it produce a severe epidemic of pulmonary disease.

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J. Hyg., Camb. (1970), **68**, 663
Printed in Great Britain

Salmonellosis in general practice. Observations of cases and their households in Enfield

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(*Received* 15 *July* 1970)

SUMMARY

Nine hundred and seventeen Salmonella infections in 580 households were confirmed during a laboratory study of diarrhoea in general practices in a large urban area during the years 1953–68. This was an annual incidence of about 2/10,000 population. Salmonellas were found in nearly 2 % of new cases of diarrhoea investigated. Plural infections were found in 36 % of the households studied and 18 % of all contacts examined were shown to be infected. Among these contacts the infection rate was higher for children (24 %) than for adults (16 %). The duration of infection was longer than 2 months in nearly a quarter of the cases followed up, and intermittent excretion was observed in one-fifth. The commonest serotype was *Salmonella typhimurium*, but its incidence in the district declined especially after 1964. *S. typhimurium* infection provoked a severer enteritis but less general symptoms than did other salmonellas. Children were more susceptible than adults to salmonella infection, to illness and to prolonged excretion, but symptoms in index cases were as severe in adults as in children.

INTRODUCTION

Salmonellosis is a common cause of food poisoning in England and its place in outbreaks has been well documented. In contrast its sporadic occurrence in general practice has been studied less.

During the years 1953–68 such a study was made among a population of approximately 250,000 in general practices in Enfield. The family doctors in the area, the local health department, and the Edmonton public health department co-operated in the diagnoses and observation of the cases. The findings are reported below.

METHODS

The first confirmed case of salmonella gastro-enteritis in any household was listed as the index case and the home was visited by a health inspector. Infected households discovered during the follow-up of contacts were also visited and listed separately. A record was made of the age, sex and occupation of each member of the household, as well as the date of any symptoms and the suspected source of infection. Each person was asked to send a faecal sample to the laboratory. First

specimens were usually received before the start of treatment. Thereafter the patients were treated by their doctors in various ways.

Patients in whose faeces salmonellas were found were asked to send weekly specimens starting 3 or more days after concluding any antibacterial treatment, until a negative result had been found. The rest of the family were then re-examined. Most families were persuaded to remain under observation until two or three consecutive negative specimens had been collected from each infected person. Negative results from unsatisfactory or dry specimens were disregarded.

Eighty per cent of the faeces specimens sent for diagnosis were examined during the first week of illness. After microscopic examination, cultures were made on MacConkey and on deoxycholate citrate agar plates and from selenite-F enrichment broth on deoxycholate citrate agar. Common salmonella serotypes were recognised by standard techniques; rarer serotypes were identified at the Salmonella Reference Laboratory.

RESULTS

Incidence of ascertained infection

During the 16 years of the study approximately four-fifths of the population of Enfield were aged 15 years or over; children were distributed fairly evenly among the age groups 0–4 years, 5–9 years and 10–14 years.

A total of 917 individual salmonella infections were recorded, a mean annual incidence of approximately 2/10,000. Of these, 539 were index cases and a further 41 infected households were found during the follow-up of contacts. These 580 households represent nearly 1 % of the households in the area.

Source of infection

Forty-three of the 539 index cases arose in five foodborne outbreaks. One of these outbreaks was traced to a convalescent carrier preparing cream cakes (Cowlard & Thomas, 1963) and the remaining four to the contamination of cooked food with raw ingredients.

Attempts to trace the source of infection of the 496 sporadic index cases were usually unsuccessful. In at least 15 instances infection was probably contracted during foreign travel, five with *S. typhimurium* and ten with other salmonellas.

Serotypes

During the period from 1953 to 1968 the commonest salmonella serotype was *S. typhimurium*, which was isolated every year and accounted for two-thirds of the total incidence. *S. enteritidis*, *S. paratyphi B*, *S. thompson*, *S. heidelberg* and *S. newport* were the next most frequently encountered; other serotypes were much rarer (Table 1).

S. typhimurium infection differed from that with other salmonella serotypes in several important respects. For purposes of comparison all the latter have been grouped together and designated 'other salmonellas'.

From 1953 there was a continued decline in the number of *S. typhimurium* isolations annually followed by a pronounced drop after 1964, although no such

trend was seen with 'other salmonellas' (Table 2). To begin with *S. typhimurium* clearly predominated, but this was not so at the end of the period. Until 1964 more than three-quarters of all salmonellas were isolated in the summer half year, but afterwards the seasonal variation was slight (Fig. 1).

Table 1. *Salmonella serotypes isolated from index cases according to 4-year periods*

Serotype	1953–6	1957–60	1961–4	1965–8	Total 16 years	Number of years isolated
S. typhimurium	132	102	93	34	361	16
S. enteritidis	7	3	4	6	20	12
S. paratyphi B	18	2	3	—	23	8
S. thompson	8	7	2	—	17	6
S. newport	1	6	1	2	10	5
S. heidelberg	—	2	11	3	16	5
30 Other serotypes	15	13	41	27	96	1–5
Total	181	135	155	72	543*	—

* Included are 4 multiple infections.

Table 2. *Index cases according to year and salmonella group*

Year	*S. typhimurium*	'Other salmonellas'	Total
1953	39	8	47
1954	39	4	43
1955	31	20	51
1956	23	16	39
1957	29	5	34
1958	32	9	41
1959	23	16	39
1960	18	3	21
1961	22	7	29
1962	25	27	52
1963	35	14	49
1964	11	13	24
1965	10	7	17
1966	10	5	15
1967	9	13	22
1968	5	11	16
Total	361	178	539

Age and sex

Of the 539 index cases, 361 were infected with *S. typhimurium* and 178 with 'other salmonellas'. The sex and age distribution differed in these two groups (Table 3). Fifty-eight per cent of *S. typhimurium* index cases were in children, whereas 58 % (also) of the index cases infected with 'other salmonellas' were in adults. This difference in the child/adult index case ratio between the two salmonella groups is highly significant ($P = < 0{\cdot}001$). There were significantly more male than female adult index cases of *S. typhimurium*, but slightly more female than male index cases infected with 'other salmonellas'.

For both *S. typhimurium* and 'other salmonellas' the highest proportion of index cases arose in the youngest population groups (Table 4). Thus there were 5·2 salmonella index cases/10,000 p.a. among the youngest children (0–4 years) and only 0·7/10,000 among adults.

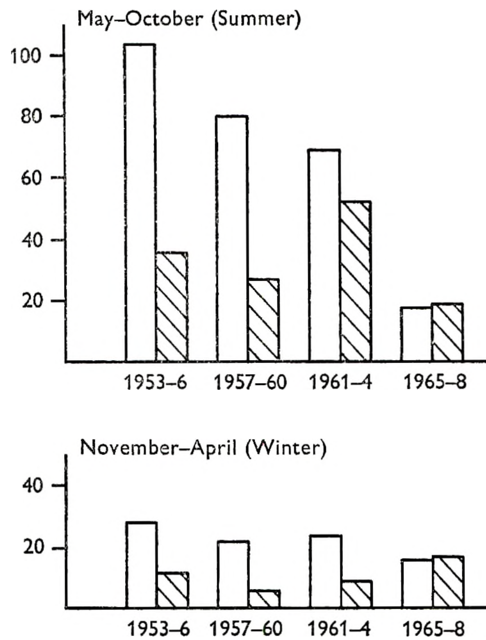

Fig. 1. Annual and seasonal incidence of index cases. □, *S. typhimurium*; ▧, 'Other salmonellas'.

Table 3. *Index cases according to sex, age group and salmonella group*

Type of index case		*S. typhimurium*	'Other salmonellas'
Child	Male	119 (33%)	39 (22%)
	Female	91 (25%)	36 (20%)
Adult	Male	87 (24%)	46 (26%)
	Female	64 (18%)	57 (32%)
Total		361	178

Severity of illness

All the 539 index cases were sufficiently ill to have called in a doctor and achieved a bacteriological diagnosis. Two distinct criteria were used to assess the relative severity of index cases. These were: early admission to hospital for medical reasons, and a record of blood or pus cells in the faeces. Admission to hospital for social reasons was not included in assessing severity.

As shown in Table 5, a slightly higher proportion of patients infected with 'other salmonellas' than with *S. typhimurium* were admitted early to hospital—17 and 13% respectively. In contrast nearly twice as many patients infected with *S. typhimurium* had blood or pus in their stools, 43% as compared with 24%, and

there was some indication that colitis persisted longer after *S. typhimurium* infection. Cells were seen in 15 of 56 (27 %) of patients infected by *S. typhimurium* whose faeces were examined in the second week of illness, but in only 3 of 32 (9 %) infected with 'other salmonellas'. *S. typhimurium* infections were thus more irritating to the epithelium of the gut than 'other salmonellas' but paradoxically a greater proportion of the 'other salmonellas' produced severe general symptoms with fever and prostration.

Table 4. *Case incidence according to age group*

Age group of index case	Total number of index cases	Mid-period population	Annual incidence/10,000 population
0–4 years	143	17,343	5·2
5–9 years	84	15,676	3·3
10–14 years	36	20,680	1·1
Child, age group unknown	22	—	—
All children	285	53,699	3·3
Adult 15+	254	220,158	0·7
Total	539	273,857	1·2

Table 5. *Severity of cases according to salmonella group, age group and sex*

Salmonella group	Index case		Admitted early to hospital	Diagnosed at home: Cells found	Diagnosed at home: No cells found	Total
S. typhimurium	Child	Male	18 (15 %)	43 (36 %)	58 (49 %)	119
		Female	12 (13 %)	48 (53 %)	31 (34 %)	91
	Adult	Male	4 (5 %)	42 (48 %)	41 (47 %)	87
		Female	12 (19 %)	21 (33 %)	31 (48 %)	64
	Total		46 (13 %)	154 (43 %)	161 (45 %)	361
'Other salmonellas'	Child	Male	5 (13 %)	9 (23 %)	25 (64 %)	39
		Female	12 (33 %)	7 (19 %)	17 (47 %)	36
	Adult	Male	6 (13 %)	12 (26 %)	28 (61 %)	46
		Female	8 (14 %)	14 (25 %)	35 (61 %)	57
	Total		31 (17 %)	42 (24 %)	105 (59 %)	178

Although as mentioned above the incidence of index cases was greatest in children, it was interesting to note that among index cases symptoms were of broadly similar severity in children and adults (Table 5).

Duration of infection

The duration of infection was taken to be from the onset of diarrhoea to the point midway between the last positive and first negative specimen of a clearance series.

Surveillance was only enforceable in those few cases carrying a specific public health risk, but most families were persuaded to co-operate voluntarily. Of the

539 index cases, 199 had three or more terminal negative specimens, 159 had two, and 66 had only one. These 66 cases have been included in an analysis of the duration of infection (Table 6) because the mathematical distribution of their durations was similar to that of the 258 cases with two or more final negatives. The last specimen examined was positive in 115 cases and six of these which were shown to be excreting salmonellas for 2 months or more are also included in the total analysed (430).

Table 6. *Duration of infection in index cases according to age and serotype*

	Age group	No. of cases analysed	< 2 weeks	2–< 4 weeks	4–< 8 weeks	2 months and longer	Lapsed from survey
S. typhimurium	0–4	90	13 (14 %)	27 (30 %)	23 (26 %)	27 (30 %)	18
	5–14	79	11 (14 %)	29 (37 %)	23 (29 %)	16 (20 %)	11
	< 15 age unknown	4	—	1	—	3	8
	15 +	120	24 (20 %)	40 (33 %)	39 (32 %)	17 (14 %)	31
	Total	293	48 (16 %)	97 (33 %)	85 (29 %)	63 (21 %)	68
'Other salmonellas'	0–4	32	6 (19 %)	6 (19 %)	10 (31 %)	10 (31 %)	3
	5–14	27	6 (22 %)	3 (11 %)	10 (37 %)	8 (30 %)	3
	< 15 age unknown	3	1	1	—	1	7
	15 +	75	19 (25 %)	17 (23 %)	22 (29 %)	17 (23 %)	28
	Total	137	32 (23 %)	27 (20 %)	42 (31 %)	36 (26 %)	41
Totals		430	—	—	—	99 (23 %)	109

The duration of infection showed no sex difference. It was significantly longer in childhood and there was a tendency for infection with 'other salmonellas' to last longer than that with *S. typhimurium* (Table 6). Infection with *S. typhimurium* persisted beyond 2 months in 30 % of preschool children, 20 % of school children and 14 % of adults; the corresponding figures for 'other salmonellas' were 31, 30 and 23 %. Twenty-three per cent of all salmonella infections lasted longer than 2 months (Table 6), 10 % (42) longer than 3 months and 4 % (17) longer than 4 months. Two patients, a man of 44 infected with *S. typhimurium* and a woman of 63 with *S. enteritidis*, were still known to be infected when they left the district more than 9 months after the onset of their illness. The woman had had six courses of treatment with different drugs and antibiotics.

Intermittent excretion was demonstrated in 108 (one-fifth) of the 539 index cases and might have been disclosed more often had longer and closer supervision been practicable. Its occurrence was not related to age, sex, or salmonella group, but probably to treatment, since in 36 cases the second specimen in a series was an intervening negative. Such specimens were liable to have been taken shortly after antibacterial therapy and were discounted for surveillance purposes. In 72 cases a negative report intervened at a later stage, and in half of them more than one such intervening negative was recorded.

Household infection

Specimens of faeces were collected from other members of the households of 94 % of laboratory index cases. Of these 508 households 181 (36 %) were found to contain further infected persons, many without symptoms. The average number of additional persons tested in each household was 3·2, being rather more for child index cases (3·7) than for adults (Table 7). Taking all ages together, the contact infection rate was 18 % for each of the two salmonella groups. Regardless of the age of the index case, children were infected more often than adults, 24 % (150/634) of child contacts being infected compared with 16 % (155/983) of adults. This difference is highly significant statistically.

Table 7. *Household infection rate according to salmonella group, age group and sex*

	Index Case		Number of households	Number of household contacts tested (all ages)	Average number tested per household	Number found positive	Rate of household infection (%)
S. typhimurium	Child	Male	115	412	3·6	72	17
		Female	86	304	3·5	59	19
	Adult	Male	82	234	2·9	35	15
		Female	60	137	2·3	33	24
	Total		343	1087	3·2	199	18
'Other salmonallas'	Child	Male	39	139	3·6	32	23
		Female	33	147	4·5	31	21
	Adult	Male	40	99	2·5	17	17
		Female	53	137	2·6	15	11
	Total		165	522	3·2	95	18

Child index cases had more child contacts than adults and household infections were more frequent when the index case was a child. Table 7 shows that for *S. typhimurium* the household infection rate was higher for female index cases, but that the reverse was true for 'other salmonellas'.

In 20 households more than one pathogen was found. These multiple infections included *Shigella sonnei*, enteropathogenic *Escherichia coli*, *Giardia lamblia*, and plural salmonella species. No less than four salmonella serotypes were recognized in one family after a holiday abroad (Thomas & Cowlard, 1965).

DISCUSSION

Since the survey in Massachusetts by Rubenstein, Feemster & Smith (1944), there have been few epidemiological reports of salmonellosis in family practice and the current study appears to be the first long-term investigation of this kind made in England. Despite the difference of time and place, our observations have been very similar to those made in the American study.

The investigation had several advantages: a reasonably stable community, the co-operation of family practices in the same district over many years, the active

participation of the public health departments throughout, and co-ordination by a single laboratory of the Public Health Laboratory Service.

Although salmonellosis is a common cause of food poisoning accounting for nearly two-thirds of such incidents (Cockburn & Vernon, 1969; Evans, 1970), the findings in Enfield confirmed reports from other areas that salmonellosis is not a common cause of diarrhoea in general practice (Thomas & Charter, 1956; Tuckman *et al.* 1962; Knox, MacNaughton, Laurence & Robertson, 1967). During the period of the study it accounted for about 2 % of diarrhoeas investigated and the findings suggested that a doctor in Enfield might have had an infected household diagnosed about once every 3 years. In the social conditions prevailing there was little evidence of case-to-case spread outside the household or even within it, since the 36 % of households in which more than one person was found to be infected may often have shared a common food source, although this was not obvious unless infected contacts had symptoms. The only outbreaks observed were all directly related to food from a common source. During the study infected children were found in schools and day nurseries without associated cases. However, this may have been because the hygiene in the Enfield nurseries was of a high standard, and nursery outbreaks are nevertheless a recognized hazard. No hospital outbreak was discovered, but they too are a serious problem when they arise. The study emphasizes that the liability to salmonella infection and to gastro-enteritis decreases with the age of the child and is least in adults. Clearly the susceptibility of infants and their tendency to prolonged infection—also shown by the study—must be taken into account in the control of human salmonellosis. It was of interest that, as reported by Rubenstein and his colleagues in 1944, the ratio of child to adult infections was greater for *S. typhimurium* than for 'other salmonellas'. The reasons can only be conjectured, but *S. typhimurium* is endemic and relatively small numbers, sufficient to produce disease in children but not in adults, may be more frequently ingested than with other salmonella species.

The continuing decline in the number of *S. typhimurium* infections in the area and their more rapid fall from 1964 onwards was associated with the loss, after 1964, of a previously marked summer predominance of all salmonellas. This was apparently a local phenomenon, since there is no evidence from the national returns of salmonella isolations made to the PHLS of a comparable reduction in summer infections. Enfield, like most of the country during the period concerned, experienced changes in food technology and distribution. Commercial and domestic refrigeration increased as did the pasteurization of milk supplies, and in 1964 the liquid egg pasteurization regulations were enforced (Statutory Instrument, 1963). In addition, from 1964 the Enfield Health Department took a very active interest in food hygiene, arranging public exhibitions as well as the regular inspection of premises and certain categories of food handlers. It may be that this local interest contributed to the difference from the national pattern.

The findings emphasize the common observation that salmonella infections are often prolonged. Half of them lasted longer than a month and 10 % persisted beyond 3 months. Excretion was more persistent in young children and after infection with 'other salmonellas'. The traditional view that a single negative

specimen is insufficient for clearance was substantiated. Sharp (1970) recorded recurrent excretion of salmonellas after three or more negatives in nearly 5 % of cases in several outbreaks and concluded that six final negatives should be required.

There is some evidence that antibiotics may prolong salmonella excretion (Dixon, 1965; Azerkoff & Bennett, 1969) and many of our cases received anti-bacterial treatment which varied with the fashions over 16 years and which may have prolonged some infections.

The study showed clearly that, although systemic symptoms tended to be more evident with 'other salmonellas' than with *S. typhimurium*, colitis was more severe with the latter. The potential severity is underlined by Boyd's (1968) observation of an acute colitis with crypt abscess formation and fibrinoid necrosis in the post-mortem examination of four patients who died of *S. typhimurium* infection. Finally, in spite of the greater susceptibility of children, but perhaps indicative of a threshold of severity at which medical help is sought, symptoms in index cases were as severe in the adult as in the child.

This study was made possible by the co-operation of General Practitioners, Medical Officers and Health Department staff in Enfield. Our thanks are due to them, to the technical staff of the one-time Edmonton Public Health Laboratory, especially the late J. H. Cowlard, and to Dr T. M. Pollock for much help with the report.

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Printed in Great Britain

The excretion of two virulent strains of African swine fever virus by domestic pigs

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(*Received* 30 *July* 1970)

SUMMARY

Daily observations were made on the excretion of African swine fever (ASF) virus by pigs infected intranasally or by contact. Two strains of virus having mean death times of approximately 3 and 6 days were used, the latter being recently isolated from a warthog.

First excretion usually occurred by the nasopharyngeal route, as early as 1 or 2 days before the onset of fever in many cases. The titres of pharyngeal and nasal swabs rose rapidly to reach mean levels of about 10^4–10^5 HAD50 at 48–72 hr. following the onset of pyrexia. Virus in the secretions of the conjunctiva or lower urogenital tract appeared later and did not attain such high levels. Faecal and urinary excretion was of relatively little significance, except in slower infections caused by the recent warthog virus.

These results are discussed in relation to the known failure of infected pigs to transmit the disease to stallmates during the first 12–24 hr. of pyrexia and also in relation to recent work on the pathogenesis of ASF in domestic swine.

INTRODUCTION

Little detailed information is available on the routes of excretion of the virus of African swine fever (ASF) by domestic pigs. Montgomery (1921) first demonstrated that the virus is not transferred through the air, in that pigs either separated by 6 in. of wire mesh, or muzzled when in the same pen as a reacting animal, do not acquire the infection. The same worker was able to infect pigs with fresh urine and faeces derived from animals which had recently died from acute ASF. Montgomery also showed that reacting pigs are not infectious to stallmates during the first 12–24 hr. of fever, an observation which has been repeated by Plowright, Parker & Staple (1968). Scott (1965) suggested that pigs are infected through nuzzling or ingestion of fomites, a hypothesis supported by recent work on the pathogenesis of the disease (Heuschele, 1967; Plowright *et al.* 1968; Colgrove, Haelterman & Coggins, 1969).

In order to elucidate the comparative importance of various routes of excretion and to explain why sick pigs are not infectious to others during the first 24 hr. of fever, a study was made of the pattern of virus excretion in domestic pigs which had been infected by natural routes with two highly pathogenic strains of virus.

MATERIALS AND METHODS

Virus strains

(*a*) The Tengani strain caused an epizootic of ASF in Malawi in 1962 (Cox & Hess, 1962) and was known to cause a rapid lethal disease in domestic swine, the mean death-time being 2·9 days (Plowright *et al.* 1968). The virus was stored at − 70° C. in the form of a 10 % (w/v) spleen suspension which, when titrated in pig bone marrow (PBM) cultures, had a titre of $10^{7\cdot4}$ HAD 50/ml.

(*b*) The Kirawira virus was recovered in PBM cultures from a warthog shot in the Kirawira Game Control Area, Tanzania, and passaged once in a domestic pig; it was characterized by a longer death-time (mean 7·0 days for 11 pigs). This virus was stored at − 70° C. in the form of portions of pig spleen which were thawed once only and used to prepare 10 % (w/v) suspensions in phosphate-buffered saline, pH 7·2 (PBS of Dulbecco & Vogt, 1954); on PBM assay this tissue had a titre of $10^{7\cdot4}$ HAD 50/g.

Experimental animals

Cross-bred, Large White: Landrace pigs of about 40–45 lb. live-weight were reared on the station. They were housed in individual pens in isolation units and their rectal temperature was recorded every morning before the sampling procedures. The 'onset of fever' was usually regarded as occurring on the day when the temperature attained or exceeded 103° F.; this time also marked the end of the incubation period.

Infection of animals

To initiate the experiments on each virus strain, two groups of two 'donor' pigs were infected by the intranasal instillation of 2 ml. of a 10 % (w/v) spleen suspension while being held in dorsal recumbency. On the third day, when the donors had a rectal temperature $\geqslant$ 103° F., a clean animal was introduced into the same pen and left there for 24 hr. before being washed liberally with running water and placed in a clean pen. Recipient pigs, when they reacted, served as donors for further clean animals and the pens were not cleaned out until the pig died.

Samples

Pigs were examined each morning before being fed, swab samples being taken from: (i) the cheek pouch of the oral cavity, between the cheeks and molar teeth; (ii) the nasal cavity, the swab was inserted 2–3 in. through the external nares; (iii) the pharynx, in the region of the palatal tonsil; (iv) the conjunctiva; (v) the vagina or prepuce, the swab was inserted 1–2 in. into the lumen.

After sampling, the swabs were immediately immersed in 3 ml. of complete PBM culture medium (Earle's balanced salt solution with 25 % normal pig serum) containing per ml.: 600 units of penicillin; 600 μg. of streptomycin; 300 μg. of kanamycin and 150 units nystatin; the fluid was expressed from the swabs within 1 hr. of collection. Faeces samples were collected from the rectum and 10 % (w/v)

suspensions were prepared in the above medium; these were centrifuged for 10 min. at 2000 rev./min. and the supernatant used as inoculum.

Blood samples were taken daily from the anterior vena cava, part being diluted with one-third the final volume of 1·5 % ethylene-diamine-tetraacetic acid: disodium salt (EDTA) in 0·7 % NaCl, whilst the rest was allowed to clot to produce serum for antibody detection.

Urine, when available, was collected at autopsy by aspiration through the bladder wall with a needle and syringe; only pigs infected with the Kirawira virus yielded urine samples. All samples were stored at about 4° C. before assay in PBM cultures.

Detection and titration of infectivity in samples

Tenfold dilutions in PBS were prepared from the samples and each dilution was inoculated in a dose of 0·2 ml. into five tubes of PBM cells. Undiluted and 10^{-1} dilutions of blood were removed after 3 hr. incubation at 37° C. and fresh medium was added to the cells. When the titre was higher than expected, retitrations were carried out from samples stored at 4° C. for periods of 2–3 weeks.

Cell cultures and infectivity end-points

Washed cell suspensions were prepared from the long bones of pigs by the method already described (Plowright *et al.* 1968). The cells were suspended in medium to a concentration of $7–9 \times 10^6$/ml. and dispensed in 1·6 ml. quantities into tubes 150 × 16 mm., which were incubated for 3–4 days at 37° C. in stationary racks before inoculation. The medium has been described in the section on 'Samples'; it included a range of antibiotics at one-third the concentration mentioned above.

A final microscopic examination for the haemadsorption and cytopathic effects of ASFV (Malmquist & Hay, 1960) was carried out on the sixth day after inoculation and end-points, as $\log_{10}$ HAD50 (50 % haemadsorbing doses) per swab, per gram of faeces or per ml. of blood, were calculated by the method of Spearmann-Karber (Dougherty, 1964).

Serology

Agar-gel diffusion precipitation (AGDP) tests and complement fixation (CF) tests were applied to all preinfection sera and to serum samples collected throughout the disease. The AGDP tests were conducted by a slide technique against a concentrated ultrasonic extract of pig kidney cells infected with the 'Uganda' strain of virus (DeTray, 1963). A similar, more dilute extract was employed in the CF test, but normal bovine serum was not included as an enhancing factor (Plowright & Staple, 1967).

RESULTS

Clinical reaction in pigs

The four pigs infected intranasally and eight infected by contact all succumbed to infection with the Tengani strain. The mean incubation periods after intranasal and contact infection were 2·9 and 3·9 days respectively and the mean death-time 2·8 and 3·3 days respectively.

Using the Kirawira virus the four donors reacted after a mean 3·1 days and nine of ten pigs infected by contact reacted after a mean of 4·4 days; the mean death-times were 5·7 and 5·8 days respectively.

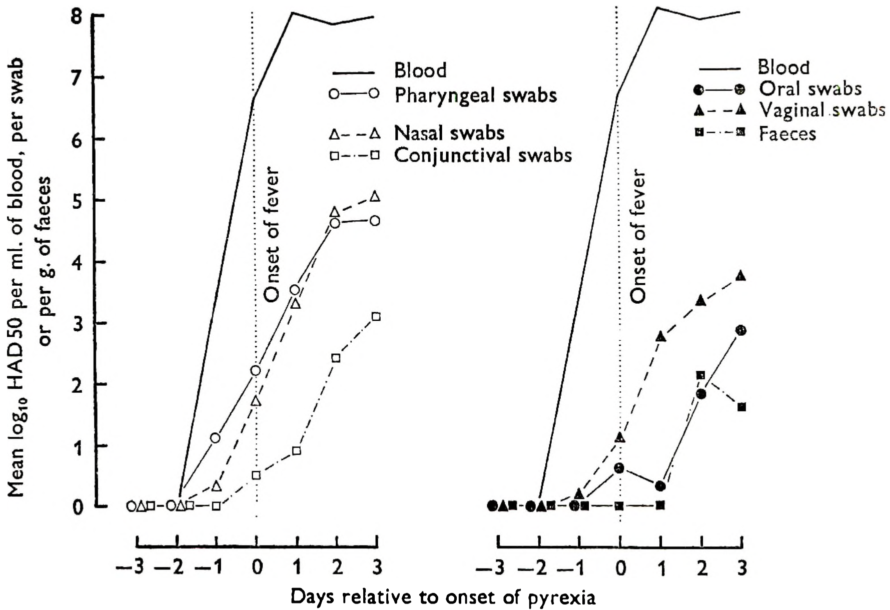

Fig. 1. The excretion of ASFV (strain Tengani) by pigs infected intranasally.

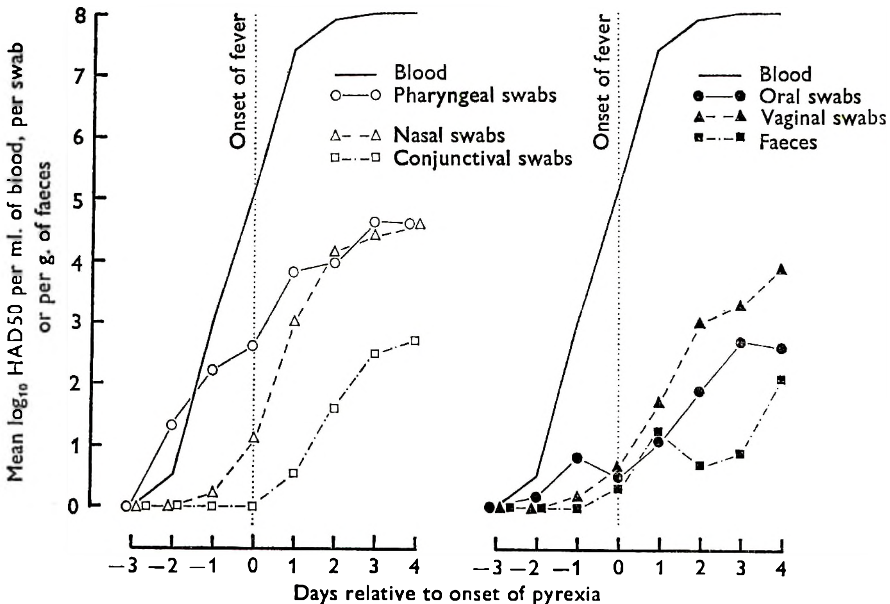

Fig. 2. The excretion of ASFV (strain Tengani) by pigs infected by contact.

The results of titrations of blood and excretions, all expressed as mean figures, are shown in Figs. 1 and 2 for the Tengani strain and Figs. 3 and 4 for the Kirawira (KWH/12) virus. All calculations were made so as to relate virus recovery to the

onset of fever because it was known that pigs are not infectious during the first 24 hr. of pyrexia and it might, therefore, have been assumed that no significant excretion of virus occurs until towards the end of this time. The figures or

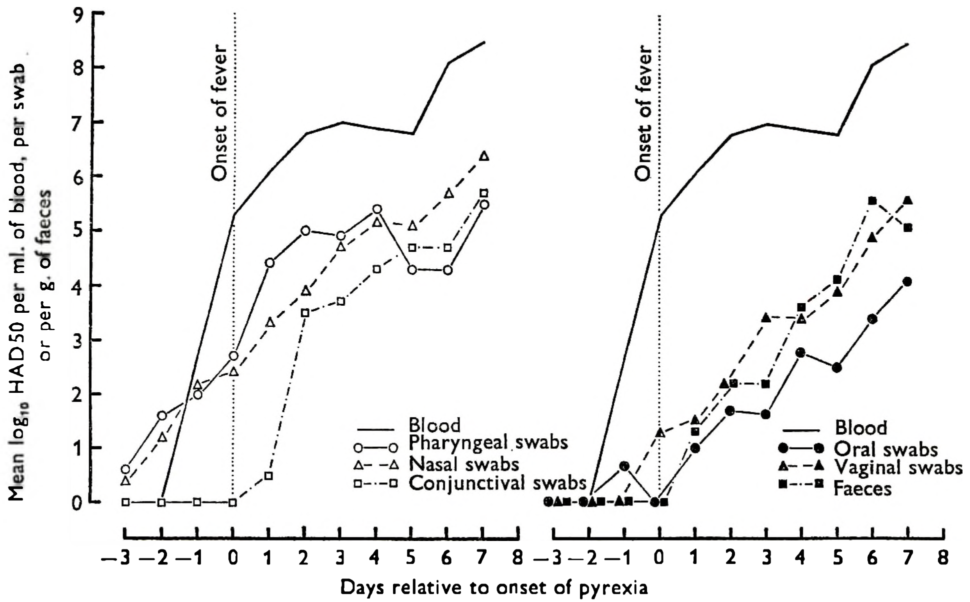

Fig. 3. The excretion of ASFV (strain KWH/12) by pigs infected intranasally.

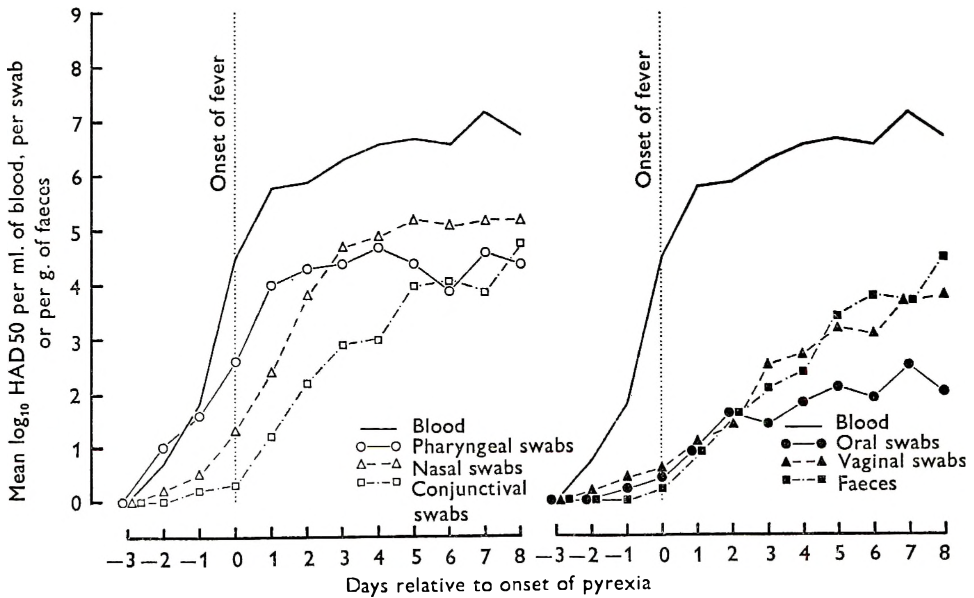

Fig. 4. The excretion of ASFV (strain KWH/12) by pigs infected by contact.

viraemia were used to prepare a reference curve for each group of pigs, indicating the time of generalization and reflecting the level of virus proliferation in the predilection tissues (Plowright *et al.* 1968; Colgrove *et al.* 1969).

Viraemia

The earliest demonstrable viraemia, 2 days before the onset of fever, occurred in two pigs infected by contact with each strain; all animals, except one Tengani contact case, were viraemic with the onset of fever. At this time blood titres for the Tengani strain varied from $10^{2\cdot0}$ to $10^{8\cdot2}$ HAD 50/ml., whereas for the Kirawira virus they were in the range $10^{2\cdot2}$ to $10^{6\cdot6}$ HAD 50/ml. An examination of the viraemia curves in Figs. 1 and 2 shows that circulating Tengani virus increased rapidly and logarithmically to reach a plateau of about 10^8 HAD 50 on the second and third days of fever. The KWH/12 virus, on the other hand, proliferated less rapidly and mean viraemia levels increased gradually throughout the course of the disease; maximum mean titres were about 10^7 HAD 50/ml. in animals infected by contact and $10^{8\cdot5}$ terminally in pigs inoculated intranasally (Figs. 3, 4).

Pharyngeal excretion

Figs. 1–4 show clearly that excretion of both strains of virus by the pharyngeal route occurred earlier and, except in the terminal stages of the disease, to higher titre than by any other pathway. Later, nasal swabs may well have included much virus derived from the nasopharynx but accumulating in the nasal passages and thus resulting in mean titres exceeding those of pharyngeal swabs.

Pharyngeal swabs first contained Kirawira virus on the third day preceding the onset of fever; this was from a single animal infected by the intranasal route. Approximately half of all the pigs infected with both strains excreted virus on the second day before pyrexia and, with the exception of one Tengani donor which excreted virus on the second day, all yielded positive swabs on the first day of fever. The quantity of virus recovered from single pharyngeal swabs varied between $10^{0\cdot9}$ and $10^{5\cdot1}$ HAD 50 up to and including the onset of pyrexia. The mean titre continued to increase up to the second or third day of fever and then maintained constant high levels, between about 10^4 and 10^5 HAD 50/swab, until death (Figs. 1–4). The titre of individual swabs also varied widely during the clinical disease, from $10^{0\cdot9}$ to $10^{6\cdot3}$ HAD 50.

Nasal excretion

Generally speaking, the recovery of ASFV from nasal swabs followed closely the pattern established for pharyngeal excretion. The relative delay in nasal excretion was more clearly evident in pigs infected by contact (cf. Figs. 2 and 4 with 1 and 3). A small quantity of virus was recovered on the day following inoculation from one of four pigs infected intranasally with the Kirawira strain; this was 3 days before the onset of fever. All of these four pigs showed virus in the nasal secretions on the day preceding first pyrexia, the titre of swabs up to this time varying from $10^{0\cdot9}$ to $10^{3\cdot1}$ HAD 50. Thereafter, the recovery of virus from single swabs varied from $10^{1\cdot7}$ to $10^{7\cdot1}$ with a progressive rise to death in mean values (Fig.3).

Nasal excretion of the Tengani strain was relatively delayed in pigs infected intranasally; only one of four pigs yielded a positive swab on the day preceding fever and all did not become positive until the second day of pyrexia. Virus titres

up to and including the first day of fever were comparable with those for the Kirawira virus.

Only one of eight animals infected by contact with the Tengani strain excreted virus by the nasal route on the day preceding fever; the behaviour of the Kirawira virus was comparable, two of nine pigs being positive at this time. About half the pigs (8/17) were nasal excretors on the first day of pyrexia, with virus titres up to this time ranging from $10^{0.9}$ to $10^{3.9}$ HAD 50/swab. All animals had become positive by the third day of fever and from this day onwards the quantity of virus in individual swabs ranged between $10^{2.9}$ and $10^{5.7}$ for the Tengani strain, and $10^{2.6}$ to $10^{6.3}$ HAD 50 for the Kirawira virus.

Conjunctival excretion

Figs. 1–4 show clearly that conjunctival excretion of ASFV first occurred 1–3 days later than by the nasopharyngeal route. It is also evident that, on average, the quantity of virus excreted by this means was considerably smaller during Tengani-strain infections than in the longer course of the disease induced by the KWH/12 virus. On and after the 5th day of clinical infection with the latter, conjunctival excretion was roughly equivalent to pharyngeal release of virus.

Conjunctival excretion of Tengani virus did not begin until the first or second days of pyrexia, in animals infected intranasally and by contact respectively; nine of twelve animals were positive by the third day of pyrexia and ten of eleven by the fourth. The mean titre of swabs increased progressively during the disease but there was considerable variation throughout, from $10^{0.9}$ to $10^{5.1}$ HAD 50/swab. The Kirawira virus appeared in the conjunctival swabs of all 13 pigs by the fourth day of the disease, a single isolation being recorded from a contact-infected animal on the first day of pyrexia. Titres of individual swabs again showed a very wide variation, from $10^{1.1}$ to $10^{6.1}$ HAD 50.

Oral excretion

Excretion of both strains of ASFV by the oral route was erratic and at a low level. Three of 17 pigs infected by contact yielded virus in oral swabs on the day preceding pyrexia, but consistent excretion by this route only occurred in the terminal stages of the disease; two of eleven pigs infected with the Tengani strain were positive on the third day of fever and 12 or 13 infected with the Kirawira virus by the sixth day of pyrexia. The amount of virus in individual swabs varied from $10^{0.9}$ to $10^{4.9}$ HAD 50; only five specimens had titres $\geq 10^{4.0}$.

Excretion from the lower urogenital tract

The lower urogenital tract was of relatively greater importance than the oral route in the excretion of ASFV; a reference to Figs. 1–4 shows that it was comparable in importance to the conjunctival secretion.

Two animals infected with each strain revealed virus in the prefebrile period with individual titres up to $10^{3.2}$ HAD 50 being recorded. All pigs infected by contact with the Tengani strain were positive by the third day of pyrexia but this situation was not reached until the seventh day in the case of the Kirawira virus.

After the onset of fever the titres of single swabs varied from $10^{1\cdot5}$ to $10^{5\cdot5}$ and $10^{0\cdot9}$ to $10^{5\cdot3}$ HAD 50 respectively for the two viruses. The mean titres rose progressively throughout the clinical disease to exceed $10^{3\cdot5}$ terminally for the Kirawira virus (Figs. 3, 4).

Faecal excretion

The figures for this route were incomplete owing to difficulties in collecting faeces in the terminal stages of the disease.

Pigs infected with the Tengani strain excreted little virus in the faeces and then in an irregular and delayed manner; virus was first detected in two 'contact' animals at the onset of fever but two of this group and one infected intranasally never became positive.

In comparison, animals infected by contact with the Kirawira virus excreted ASFV in the faeces from the first day of fever onwards but all pigs did not become positive until the sixth day; all four animals infected intranasally were faecal excretors by the third day of pyrexia. The quantity of virus in the faeces varied for this strain from $10^{1\cdot4}$ to $10^{5\cdot8}$ HAD 50/g., the mean titres later in the disease exceeding those for the Tengani virus by 2 to 2·5 $\log_{10}$ units.

Urinary excretion

Since animals dying after infection with the Tengani strain almost invariably had an empty, contracted bladder, no figures were obtained for this virus. Three of four Kirawira 'donors' had ASFV in the urine at death, with titres varying between $10^{3\cdot0}$ and $10^{5\cdot0}$ HAD 50/ml.; six of nine animals infected by contact also had virus in the urine, varying in quantity from $10^{0\cdot8}$ to $10^{4\cdot2}$ HAD 50/ml.

The antibody reaction in acute ASF

No precipitating or complement-fixing antibodies were detected in serum samples collected throughout the course of the disease.

DISCUSSION

The results reported in this paper show unequivocally that two strains of ASFV were excreted by many infected pigs 1 or 2 days before the onset of fever, particularly by the nasopharyngeal route. It is necessary to remark, however, that all pigs did not become excretors of detectable amounts of virus until the second day of pyrexia, i.e. at least 24 hr. after the onset of fever. It was also evident that the quantity of virus excreted, reflected here in the mean titre of swabs, usually remained small during the first day of the clinical reaction. Failure of the virus to spread to susceptible pigs in close contact with infected animals during their first 12–24 hr. of pyrexia must, therefore, be due to inadequate degrees of contamination of the environment, including food and water, rather than the absence of virus excretion, as suggested by Plowright *et al.* (1968). The dose of ASFV necessary to infect pigs by natural routes has not been determined accurately, but Heuschele (1967) quoted Maurer *et al.* (1954, unpublished results) as showing that at least $10^{5\cdot0}$ parenteral ID 50 of pig-tissue virus were necessary to infect pigs

orally. Using warthog-tissue virus, Plowright, Parker & Peirce (1969) found that $10^{6.1}$ HAD50 did not infect orally, whereas doses of about 10^3 to 10^4 HAD50 did infect a proportion of pigs when administered intranasally.

The early recovery of ASFV from the nasopharynx of naturally infected pigs supports the contention of several groups of workers that first proliferation of virus commonly occurs in the pharyngeal area, either the palatal tonsil (Heuschele, 1967; Colgrove *et al.* 1969; A. Greig, to be published) or the retropharyngeal mucosa (Plowright *et al.* 1968). Virus in oral swabs can also possibly be regarded as arising from the pharyngeal area, since there are very seldom macroscopic lesions of the mouth epithelia in ASF and the amount of virus in the oral cavity was consistently lower than that in the pharynx.

As already observed, virus in the nasal cavities may well have been derived, at least partially, from the nasopharynx. In addition, there can be little doubt that oedema fluid or froth from the trachea and bronchi, which sometimes appears at the nostrils in the terminal stages of the disease, could also contribute to the virus recovered from both the pharyngeal and nasal cavities; the titre of lung tissue in acute ASF due to the Tengani strain of virus is commonly greater than 10^7 ID50/g. (Plowright *et al.* 1968; Colgrove *et al.* 1969), whilst interstitial and alveolar oedema are frequent gross lesions.

Whereas the major source of contamination of the environment is the nasopharynx in the early course of the infection, conjunctival and genital discharges may contribute significantly at a later stage, particularly in more prolonged forms of the disease, as produced by recent warthog viruses. Our data on virus in the urine are incomplete but do not suggest that large quantities of virus are excreted by this route, in spite of the fact that considerable numbers of petechial or larger haemorrhages are usually present in the cortex, medulla and pelvis of the kidneys, as well as less frequently in the urinary bladder. It is, therefore, surprising to note that Montgomery (1921) had no difficulty in infecting pigs with fresh urine administered orally.

Very small quantities of virus were excreted in the faeces of pigs infected with the Tengani strain of ASFV and high mean levels ($\geqslant 10^4$ HAD50/g.) were not attained in animals infected by the Kirawira virus until the last day or two preceding death. The first observation is in keeping with the fact that the mean titres of the washed mucosae of the small and large intestines of pigs infected with the Tengani virus were always one or two $\log_{10}$ units lower than those of the blood at the same time (Plowright *et al.* 1968). The later rise of faecal titres in pigs infected with a 'slower' strain of virus may have been a result of the release into the faeces of high-titre blood from haemorrhages in the intestinal mucosae. Diarrhoea and dysentery was sometimes observed in advanced cases of the disease and in these the total quantity of virus excreted in the faeces must have been very considerable.

We are grateful to Messrs C. T. Perry, W. J. Doughty, R. W. Kapaata, H. Agili and D. Waweru for their able technical assistance, and to Mr C. S. Rampton who produced the Figures.

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J. Hyg., Camb. (1970), **68**, 683
With 3 plates
Printed in Great Britain

Serotypes of sex pili

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(*Received* 4 *August* 1970)

SUMMARY

There are situations in which direct observation of the attachment of antibody molecules may be the simplest method of detecting antigen-antibody reactions. Applied to the study of the sex pili determined by a number of transmissible bacterial plasmids, the method has distinguished four serotypes in the F-like class and two in the I-like class. Antibody was usually attached haphazardly to the pili; however, in a few cases a regular periodicity could be observed. When few antibody molecules were attached, they could frequently be individually resolved and in certain antibody-pilus combinations large antibody molecules, tentatively identified as IgM, could be seen to predominate.

INTRODUCTION

The ability of *Escherichia coli* K12 to conjugate and act as a genetic donor is due to the presence of an extrachromosomal genetic element named F, for 'fertility' (Cavalli, Lederberg & Lederberg, 1953; Hayes, 1953) or 'sex factor'. The first indication that F determined the synthesis of a structural component of the donor cell was the discovery by Ørskov & Ørskov (1960) of an F-specific surface antigen, which made F^+ bacteria agglutinable by homologous antiserum from which all agglutinins for F^- bacteria had been removed. When F was later shown to determine the special type of filamentous appendage named F-pilus (see Brinton, 1965), it was here the F-specific antigen detected by agglutination was seen by electron microscopy to be located (Ishibashi, 1967; Lawn, Meynell, Meynell & Datta, 1967).

Since the discovery of F, a number of analogous genetic elements have been found in plasmids such as the Col factors responsible for transmissible colicin production (see Fredericq, 1963) or R factors responsible for transmissible resistance to antibiotics (see Watanabe, 1963). These sex factors also determine sex pili as can be demonstrated with derepressed mutants, pilus production being regulated by a repressor in the wild-type (see Meynell, Meynell & Datta, 1968; Meynell & Cooke, 1969). The majority of sex factors belong to one or other of two major classes related either to F or to the sex factor of ColI (Lawn *et al.* 1967). F-like and I-like sex pili can be distinguished morphologically, by the existence of donor-specific phages specific for each and by serological relationships within, but not between, each class (Sekijima

& Iseki, 1966; Lawn *et al.* 1967; Nishimura, Ishibashi, Meynell & Hirota, 1967; Kétyi & Ørskov, 1969). There are, however, minor differences between sex piil belonging to the same class, and the present paper reports a serological analysis which has distinguished four serotypes in the F-like class and at least two in the I-like class. The tests were made by observing antibody bound to the sex pili by electron microscopy; in this way, difficulties of agglutination tests with unstable bacterial suspensions were avoided. Bacteria carrying F or ColV form uniform suspensions in a medium of suitable pH, but the pili of bacteria carrying the other sex factors caused the bacteria to aggregate spontaneously. Moreover, agglutination tests for sex pili with whole organisms require antisera free from antibodies to other bacterial components such as cell wall or flagella. Such antibodies do not interfere with electron microscopical observation where the reactions of the serum with the sex pili can be distinguished morphologically from reactions with flagella or bacterial surface.

METHODS

Plasmids

The plasmids examined are listed in Table 1 with references to their isolation, and their classification into the two major groups, F-like and I-like (Lawn *et al.* 1967). Only cultures in which a large proportion of the bacteria produce sex pili could be tested. Wild type F and ColV produce sex pili constitutively but it was

Table 1. *Plasmids*

Plasmid	Sex factor class	References
F	F-like	Hayes, 1953; see Brinton, 1965
ColV–K94	F-like	Kahn & Helinski, 1964; MacFarren & Clowes, 1967
R 100	F-like	Egawa & Hirota, 1962; Nishimura *et al.* 1967
R1	F-like	see Meynell *et al.* 1968
R136	F-like	see Meynell *et al.* 1968
R192	F-like	see Meynell *et al.* 1968
R538–1	F-like	Romero & Meynell, 1969
R64	I-like	see Meynell *et al.* 1968
R144	I-like	see Meynell *et al.* 1968
R163	I-like	see Meynell *et al.* 1968
R538–2	I-like	Romero & Meynell, 1969
ColI–P9	I-like	Ozeki, Stocker & Smith, 1962; Meynell & Lawn, 1967*a*
ColEla	I-like	Lewis & Stocker, 1965; Meynell & Lawn, 1967*b*

necessary to use derepressed mutants of the other factors. There is no reason to believe that the structure of the sex pilus in these mutants differs from the wild-type, for no difference between individual mutants could be detected in cases where more than one derepressed mutant of the same factor was available for examination. Reference to any particular sex factor other than F and ColV implies a bacterium carrying a derepressed mutant of that factor. The sex factors

R 538–1 and R 538–2 are given suffixes because they are two separate R factors which together account for the infectious drug resistance of a clinical isolate, R 538 (Romero & Meynell, 1969).

Bacteria

The numbers of sex pili produced with a given sex factor depend to a substantial extent on the bacterial host. Wherever possible, the sex pili were examined in the *fim*⁻ (*pil*⁻) mutants, RC 22 or RC 24, of *E. coli* K 12 strain 945 which are unable to produce common pili (Maccacaro, Colombo & Nardo, 1959). Derepressed R 100 (R 100–1: Egawa & Hirota, 1962) produced very few sex pili in either RC 22 or RC 24 and was therefore examined in another K 12 strain, J 5–3, which was *pil*⁺. Since the bacteria were generally grown on solid medium, which discourages production of common pili (Duguid & Wilkinson, 1961), these interfered very little with the tests. In general, derepressed R factors lead to the formation of many more sex pili than either F or ColV and when F pili were scarce in RC 22 or RC 24 they were sometimes more abundant in a *pil*⁺ strain of *Salmonella typhimurium* carrying an F-*lac* factor (SA 197, kindly provided by Dr K. Sanderson). ColI and ColEla sex pili were examined in both J 5–3 and *S. typhimurium* LT 2.

Both for the production of antisera and for the serological tests, the bacteria were grown on nutrient agar plates incubated at 37° C. for 18–24 hr. Culture on agar improved the yield of R pili and as many F pili were obtained on agar as in broth. Reactions with common pili were examined in broth cultures of strain J 5–3 incubated at 37° C. for 24 hr. without shaking (Duguid & Wilkinson, 1961).

Antisera

For all the antisera except EM159, EM168, EM157, EM165 and EM169, which were produced with formalinized cultures and kindly provided by Dr Naomi Datta, rabbits were inoculated intravenously with live cultures containing about 5×10^8 bacteria/ml. Two, or in a few cases more, rabbits were used for each plasmid. The numbers in the tables refer to individual rabbits. Antibacterial antibodies were removed by absorption with the host bacterium, and sex pilus antibody with bacteria carrying the appropriate plasmid. The absorbed sera were Seitz filtered, which was effective in removing residual fragments of sex pili of the absorbing strain but slightly decreased the antibody titre.

Serological tests

The serological test entailed the direct observation of unlabelled antibodies bound to the antigen (sex pilus), according to the method of Lawn (1967). The bacteria were suspended in distilled water at a concentration of about 2×10^8/ml. and one drop of suspension was added either to one drop of unabsorbed antiserum diluted 1/20 in distilled water, to give a final concentration of 1/40, or to one drop of absorbed antiserum whose effective concentration after dilution and Seitz filtration was nearly twofold higher. The mixtures, on sheets of 'Parafilm', were left at room temperature for $\frac{1}{2}$–3 hr. The amount of antibody bound increased considerably during the first 10 min. after mixing, but changed little after the

first $\frac{1}{2}$ hr. About 0·005 ml. of the mixture was then transferred to a 5 mm. diameter disk of membrane filter ('Sartorius', pore size 0·05 mμ) and unbound antibody was removed by suction. The organisms were then washed by dialysed broth followed by distilled water; transferred to an electron microscopy specimen grid (coated with formvar and carbon) by floating the grid on the final drop of water and sucking it down on the filter; and, finally, stained with a 1 % solution of uranyl acetate in distilled water. Hydrophobic grids (coated with carbon in an evaporation unit lacking a liquid nitrogen trap to the diffusion pump) adsorbed fewer bacteria but gave a cleaner background because they adsorbed less unbound antibody. For investigating a non-random form of antibody attachment observed in certain antigen–antiserum combinations, other negative stains and methods of fixation were tested, which are mentioned in the relevant section.

Grading the antigen–antibody reaction

The degree of antibody binding after exposure to a fixed dilution of serum was assessed according to an arbitrary grading system. An end-point method with graded dilutions of serum proved to be impracticable because of the difficulty of establishing a precise end-point (see Non-uniform attachment of antibody). Five grades ranging from 0 to + + + + (maximal reaction) were recognized (Pl. 1, fig. 1). These grades have not been related to titres obtainable by standard immunological procedures such as agglutination; however, in electron micrographs of comparable tests with flagella (Feinstein & Munn, 1966), the reaction shown at the agglutination end-point would have been graded 0 to + in the scheme used here. The reliability of the method was established by repeated tests of the same serum and antigen, for the results agreed within one grade even if they were separated by intervals of several months. Many of the antisera were sufficiently potent to give a + + to + + + reaction at a dilution of 1/8000.

RESULTS

F-like factors

The results with unabsorbed antisera confirmed the previous conclusion that F-like sex factors are serologically related to one another (Lawn *et al.* 1967). Nevertheless, there was such a variety of reactions that the pili determined by different plasmids could in many cases be distinguished (Table 2). The differences between the pili were emphasized when cross-absorbed sera were used (Table 3). The results indicated four serotypes in the F-like class: the first is the F serotype, represented by F and ColV–K 94; the second and third are the R 538–1 and R 1 serotypes, each represented by only one member; and the fourth is the R 100 serotype, represented by R 100, R 136 and R 192. Cross-absorbed sera did not distinguish between the pili determined by F and ColV–K 94, nor between those determined by R 100, R 136 and R 192. The R 538–1 and R 1 serotypes were more closely related to one another than were any other pair of serotypes because absorption of anti-R 1 serum with R 538–1 pili removed all antibody reacting with R 1 pili. However, the two factors were not identical because absorption of anti-

Table 2. *Reactions of F-like sex pili with unabsorbed antisera*

Antiserum to ...	F Number				ColV-K 94 Number		R 1 Number			R 538–1 Number		R 192 Number		R 136 Number		R 100 Number	
	EM 159	EM 168	265	266	EM 157	EM 165	EM 169	257	264	97	100	96	99	214	215	216	217
Antigen																	
F	4*	3–4	4	4	4	4	1	2–3	3	3	3–4	1–2	2–3	1–2	2–3	2–3	1–2
ColV-K 94	4	3–4	4	4	4	3–4	0–1	2–3	2–3	3	4	1–2	2–3	1–2	3	2–3	1–2
R 1	2–3	1–3	0	0	0–1	0–1	4	4	4	4	4	0	0	0	0–1	0	0
R 538–1	2–3	0–3	1–2	0–2	2–3	0–3	4	4	4	4	4	0	0–2	0	0–2	1–2	0–1
R 192	0–1	0	1–2	1–3	1–2	1–2	0	0	1–2	2–3	1–3	4	4	4	2–4	2–4	4
R 136	0–2	0	0–2	1–2	0–2	0–2	0	0	0–1	1–3	1–3	4	4	4	3–4	3–4	4
R 100	0–2	0	0–2	1–2	0–2	1–2	0	0	0	1–3	1–3	4	4	4	2–4	3–4	4

* For the system of grading, see Methods (the + symbols have been omitted).

Table 3. *Reactions of F-like sex pili with cross-absorbed antisera*

Antiserum to ...	F Number EM 168			F Number 265		ColV-K 94 Number EM 165			R 1 Number EM 169			R 538–1 Number 97				R 192 Number 99				R 136 Number 214		R 100 Number 217	
Absorbed with ...	ColV	R 1	R 538–1	R 538–1	R 136	F	R 1	R 538–1	F	ColV	R 538–1	F	ColV	R 1	R 192	F	R 538–1	R 136	R 100	R 192	R 100	R 192	R 136
Antigen																							
F.	0	4	1–3	4	4	0	4	4	0	0	0	0	0	1–2	4	0	1–2	—	—	—	—	—	—
Col V-K 94	0	4	2–4	—	—	0	4	4	0	0	0	0	0	0–2	—	—	—	—	—	—	—	—	—
R 1	0	0	0	—	—	0	0	0	4	4	0	4	1–3	0	4	—	—	—	—	—	—	—	—
R 538–1	0	0	0	0	0	0	2–3	0	4	4	0	4	2–3	0–3	4	0	0	—	—	—	—	—	—
R 192	—	—	—	0	0	—	0	0	—	—	—	0	0	0	0	4	4	0	0	0	0	0	0
R 136	—	—	—	—	0	—	—	—	—	—	—	—	—	—	—	4	4	0	0	0	0	0	0
R 100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0	0	0	0	0

Table 4. *Reactions of I-like sex pili with unabsorbed antisera*

Antiserum to ...	R 64 Number		R 144 Number		R 163 Number		R 538–2 Number	
	113	114	160	172	116	117	108	110
R 64	4	3–4	3–4	3–4	3–4	1–2	4	3–4
R 144	3–4	3–4	3–4	3–4	3–4	1–2	4	2–3
R 163	3–4	3–4	3–4	3–4	3–4	2–3	4	3–4
R 538–2	3–4	3–4	3–4	3–4	3–4	1–3	4	3
ColI	4	3–4	3–4	3–4	3–4	2–3	4	3–4

Table 5. *Reactions of I-like sex pili with cross-absorbed antisera*

Antiserum to ...	R 64 Number 113			R 144 Number 172			R 163 Number 117			R 538–2 Number 110			
Absorbed with ...	R 144	R 163	R 538–2	R 64	R 163	R 538–2	R 64	R 144	R 538–2	R 64	R 144	R 163	I
R 64	4	4	4	0	0	0	0	0	0	0	0	1	0
R 144	0	0	0	1–3	0	0	2–3	0	0	4	0	0	0
R 163	0–1	0	0	2–3	0	0	4	0	0	4	0	0	0
R 538–2	0	0	0	2–3	0	0	3–4	0	0	4	0	0–1	0
ColI	0–1	0–1	0–1	3	—	—	3–4	—	—	4	—	—	0

R 538–1 serum with R 1 pili did not remove all the activity for R 538–1 pili and, further, there was present in a serum prepared against ColV–K 94 a small minority of antibody molecules which reacted with R 538–1 pili and not with R 1 pili.

I-like factors

There was evidently less antigenic diversity among I-like than among F-like sex pili, for each reacted indistinguishably from all the others in tests with unabsorbed antisera (Table 4). The pili of ColI and the I-like sex factor of ColEla, which were also tested, reacted similarly. Even serum 117, prepared against R 163, which had a relatively low titre of antibody and gave submaximal reactions at the standard dilution, did not distinguish between its homologous and heterologous antigens. With cross-absorbed sera, R 64 pili could be distinguished from the pili of R 144, R 163 and R 538–2, which, if one discounts minor reactions which may have been due to inadequate absorption of the sera, were probably similar to one another (Table 5). The reactions of ColI and ColEla pili placed them with the latter group. These I-like sex pili are therefore provisionally divided into two serotypes only, an R 64 serotype and another which is called the ColI-R 144 serotype (Table 6). The status of the second type may need revision when mirror tests are made with antisera to the Col factor pili.

Table 6. *Serotypes of sex pili*

	F-like			
Serotypes	F	R 1	R 538–1	R 100
Members	F ColV-K 94	R 1	R 538–1	R 100 R 136 R 192

	I-like	
Serotypes	R 64	ColI-R 144
Members	R 64	R 144 R 163 R 538–2 ColI ColEl a

Tests for cross-reactions between F-like and I-like sex pili: spurious heterologous reactions

The lack of antigenic relationship between F-like and I-like sex pili (Lawn *et al.* 1967) was confirmed with a more extensive range of antisera. All the antisera prepared against F-like pili were tested with R 64 and R 144 and all those against I-like pili with F, R 1 and R 192.

The sera were tested at 1/10 after absorption of antibacterial antibodies. However, at this low dilution there was a certain amount of particulate material which could, by masking a weak positive reaction, make it indistinguishable from a negative reaction. Five sera, nevertheless, gave reactions that could be unambiguously graded as + + or more. Three of these (numbers 160, 116 and 108) had been prepared against bacteria carrying I-like pili (R 144, R 163 and R 538–2,

respectively) but reacted also with F-like pili (F and either R1 or R192). The other two (EM159 and 100) were prepared against F-like pili (F and R538–1, respectively) but reacted also with the I-like pili of R64 and R144. When the sera were retested at the standard 1/40 dilution, the activities of numbers 160, 108 and 100 could no longer be detected, and those of numbers 116 and EM159 could be graded as only + to + +. Although this result suggested an antigenic relationship, even if only a minor one, it proved to be due to antibodies other than those directed against the immunizing antigens. In each case, absorption of the serum with the strain used for immunization removed all reactivity with pili of its own class (F-like or I-like) but not the other class.

To test whether rabbit sera contained 'natural' antibodies to sex pili, sera from 11 rabbits which had been used for purposes unconnected with the production of enterobacterial antibodies were tested against the same representative set of sex pili of both classes. Three of the 11 sera gave positive reactions with the F pili at 1/10 and two of these at 1/40 (graded 0 to + and + to + +).

All the sera, including these 11 as well as the 25 sex pilus antisera, were tested at 1/10 with J5–3F^-R^- for antibody against *E. coli* common pili. The R100 antisera, numbers 216 and 217, necessarily had anti-common pilus activity because the animals had been immunized with the R factor in the pil^+ strain J5–3. Two of the 34 other sera, numbers EM168 and 214, also contained common pilus antibody, although in relatively smaller amounts.

'Terminal knobs'

Lawn (1966) suggested that the knob sometimes seen at the tip of a sex pilus is cell-wall material. Detached cell-wall fragments evidently may become attached to the distal ends of sex pili, because they react with antibody to the cell-wall but not to the pilus (Pl. 2, fig. 6). Nevertheless, not all terminal knobs reacted in this way, for some were seen to adsorb pilus-specific antibody (Pl. 2, fig. 5). This observation, together with the fact that knobs are more frequent on pili with structural mutations (Meynell & Aufreiter, 1969) and can also be produced by treatment with low concentrations of the detergent sodium dodecyl sulphate (unpublished observations) suggests that many knobs are simply assemblies of pilus subunits less ordered than those forming the sex pili proper.

Type and distribution of antibody molecules

Non-uniform attachment of antibody

When the concentration of antiserum was sufficiently high, all the pili were uniformly and maximally (+ + + +) coated with antibody molecules. With lower concentrations, the pattern of attachment of the fewer antibody molecules depended on the particular antigen-antibody system. At one extreme, the variation in distance between individual antibody molecules was not significantly greater than to be expected from random attachment to a uniform antigen. At the other, the density of packing varied considerably from one region of a pilus to another (Pl. 2, fig. 2) and to an even greater extent between different pili in the same

culture. This might have been due to attachment, not of individual antibody molecules, but of aggregates resulting, for instance, from the presence of free pili subunits in the preparations. Nevertheless, along the sections of pilus where the largest amounts of antibody were attached, the increase in diameter of the pilus due to the attached antibody was relatively constant and compatible with the estimated thickness of a monolayer of globulin molecules. Where, as occasionally occurred, there were recognizable aggregates of antibody molecules, these were associated with an increase in diameter which was both greater and more uneven.

Non-uniform distribution of antibody was characteristically most pronounced in weak reactions of antigen with heterologous antibody, but with certain of the antigens, particularly R538-1, it occurred with many antisera. It was generally absent in homologous reactions with unabsorbed antisera. Its occurrence in the tests at standard dilution is apparent from the way in which the reactions are recorded in Tables 2–5 where a range, such as 0 to + + or + to + + +, spanning more than one grade of reaction signifies greater or lesser degrees of non-uniformity. Reactions which were non-uniform on one occasion were always non-uniform when repeated, although the degree of non-uniformity might vary.

It is possible that the non-uniformity was due to the preparative procedures for electron microscopy. Thus, antibody might have been removed by the highly acid uranyl acetate stain. The preparations were therefore fixed with formalin or glutaraldehyde to produce cross-linking within antigen–antibody complexes before the uranyl acetate was applied. However, the distribution of antibody remained non-uniform, as before, and was equally so when uranyl acetate was replaced with neutral stains such as neutral solutions of potassium phosphotungstate or potassium silicotungstate. The image of free antibody molecules is not the same with uranyl acetate as with these other stains (Pl. 3, fig. 9) but the latter are greatly inferior to uranyl acetate as stains for bound antibody molecules. Non-uniformity was equally evident when broth, 0·85 % saline or distilled water was used as diluent.

Another possible cause of non-uniformity is slow or inadequate penetration of antibody into clumps of pili, so that all the available antibody is bound to those parts of the pili that are exposed. Accordingly, the antigen concentration was varied over a tenfold range with a constant dilution of antiserum and antigen-antiserum mixtures were lightly sonicated to disperse bundles of pili before making a further addition of antiserum. None of these procedures altered the antibody distribution in any way.

Types and arrangements of antibody molecules

In maximal (+ + + +) reactions, the antibody molecules were usually attached haphazardly. However, in a few cases, a regular periodicity was observed which was sometimes transverse (Pl. 2, fig. 3) and sometimes diagonal (Pl. 2, fig. 4) to the long axis of the pilus. The reason for this occasional periodicity is not clear. It might reflect a repeating structural pattern of the antigen underneath, that cannot be resolved in the untreated pilus, or a regular arrangement of antibody molecules entailed by very close packing on the antigen surface.

In weak reactions, individual antibody molecules could sometimes be seen to resemble morphologically molecules of IgM or IgG (Green, 1969). For the most part, the antibody consisted of small molecules, like IgG (Pl. 3, fig. 7), as was to be expected in antisera raised by a prolonged course of immunization (Uhr & Finklestein, 1963). In a few antigen-antibody combinations, however, the predominant antibody molecule was large with the star- or crab-shaped appearance of IgM (Pl. 3, fig. 8, 9).

DISCUSSION

Contrary to what was originally supposed, the ability to transfer genetic information by conjugation is relatively frequent among the enterobacteriaceae. Whatever the nature of sex factors and whether or not they constitute a single biological group in which all the members have evolved from one another, it is clear that they comprise at least two classes producing dissimilar sex pili subject to independent systems of regulation. In addition, four serotypes are distinguishable within the F-like class and two within the I-like class. Antigenic differences have also been reported between the sex pili determined by the ColB factor and both F and R 1 (Kétyi & Ørskov, 1969). Within each of the two classes, the genes determining the various pili serotypes probably bear the same relationship to one another as, for instance, the H 1 genes of different salmonellae, which have similarly been shown to share a common system of regulation (Lederberg & Iino, 1956; Pearce & Stocker, 1967).

Most transmissible plasmids have been recognized because the sex factor is linked to other genes, collectively named 'somatic' (see Novick, 1969), determining bacterial characters such as colicin production or antibiotic resistance. The use of the names of the somatic genes to name the plasmid is a logical practice but experience indicates that these names do not reliably identify the sex factor itself, partly because of the readiness with which the somatic genes can be lost or exchanged by recombination between one plasmid and another (see Meynell *et al.* 1968). Even within the present relatively small sample, there were three cases where more than one plasmid determined a sex pilus of the same serotype. The simplest assumption is that in each case, different somatic genes were linked to the same sex factor. A difficulty arises therefore in referring to the sex pili serotypes but it seems nevertheless preferable, in the present state of knowledge, to retain terms such as 'R100 serotype' rather than introduce entirely new letters or numbers. Ultimately it may prove desirable to define serotypes more accurately by combinations of symbols in the same way as these are used for flagellar and other bacterial antigens.

The 'natural' anti-pilus antibodies in some rabbit sera require comment. Most wild-type sex factors are normally repressed and therefore produce more than 1000-fold less pilus antigen than even the small amounts present with the derepressed mutants used for producing the antisera. Nevertheless, these amounts are evidently sufficient to elicit natural antibodies during the course of the rabbit's life, unless some other hitherto unrecognized antigen related to sex pili is implicated. 'Natural' antibodies to *E. coli* common pili were not any more frequent

than antibodies to sex pili, although the animals were likely to have been exposed to much greater numbers of common pili in the environment than to sex pili.

The non-uniform attachment of antibody molecules sometimes observed is difficult to explain if, as is believed, pili are composed of uniform subunits and if individual antibody molecules are attached independently of one another.

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EXPLANATION OF PLATES

All are electron micrographs negatively stained with uranyl acetate. The calibration bars represent 100 nm.

PLATE 1

Fig. 1. Grading of antigen–antibody reactions (see Methods).

PLATE 2

Fig. 2. The non-uniform reaction between serum 97 (anti-R 538–1) and an R 538–1 pilus. There is a large variation in distance between individual antibody molecules along the pilus.

Fig. 3. Regular transverse periodicity shown when serum 97 (anti-R 538–1) reacts with R 100 pili.

Fig. 4. Regular diagonal periodicity shown when serum 113 (anti-R 64) absorbed with R 538–2 reacts with R 64 pili.

Fig. 5. Reaction of sex pili with anti-pilus serum containing no cell-wall antibody. The terminal knob (arrow) reacts in the same way as the pilus.

Fig. 6. Reactions of flagella and a free cell-wall fragment (single arrow) with antiserum. The sex pilus itself does not react with this antiserum but a positive reaction is shown by its terminal knob (double arrow).

PLATE 3

The calibration bar on Fig. 9 also refers to Figs. 7 and 8.

Fig. 7. Small immunoglobulin molecules attached to a sex pilus. Note the pairs of parallel rod-shaped structures (arrows).

Fig. 8. Large star- or crab-shaped immunoglobulin molecules (arrows) attached to sex pili.

Fig. 9. Human IgM immunoglobulin molecules negatively stained with uranyl acetate, for comparison with Fig. 8. (The sample of IgM immunoglobulin was kindly provided by Professor R. A. Kekwick.)

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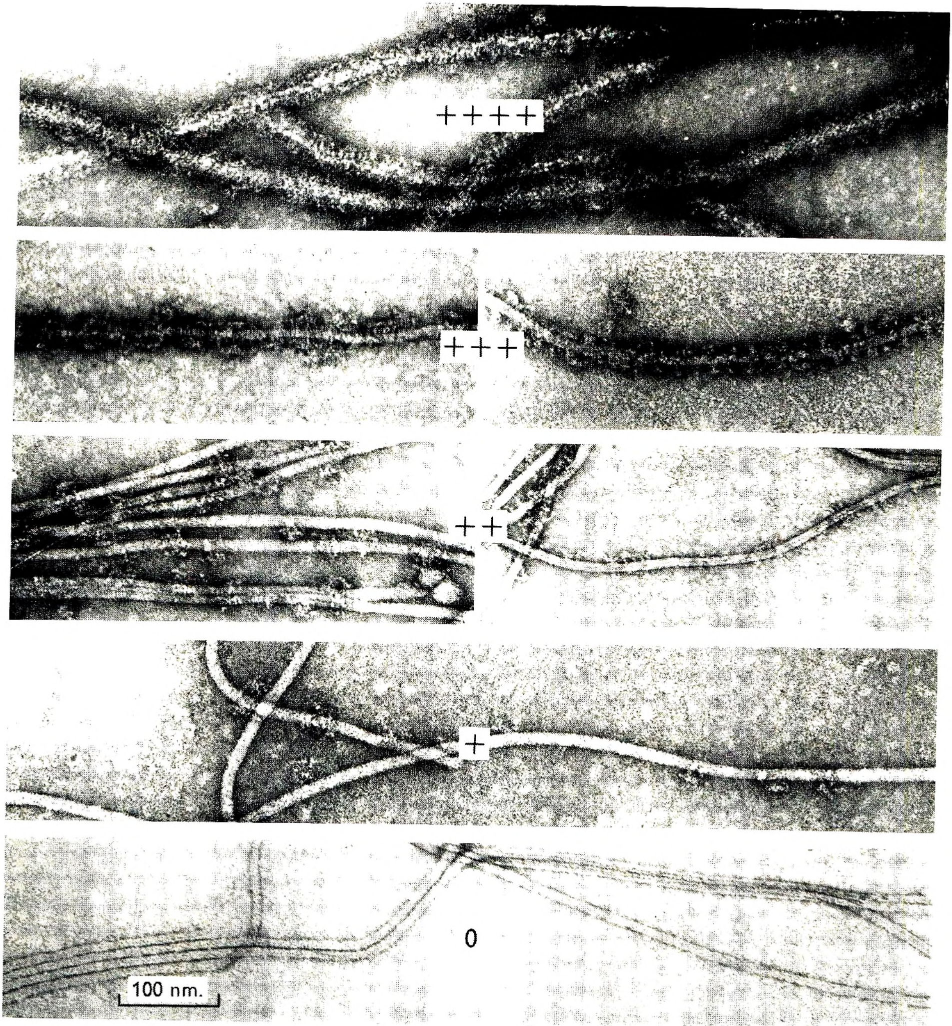

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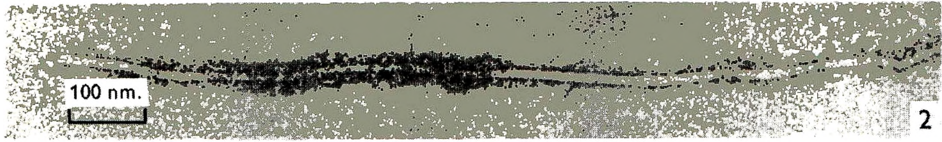

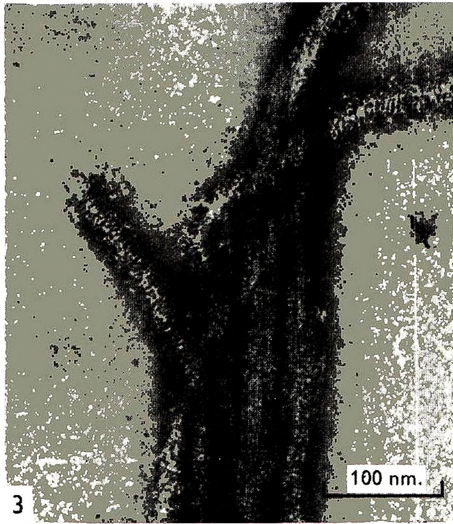

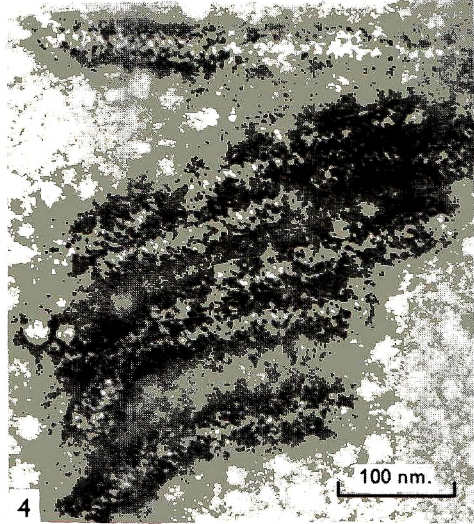

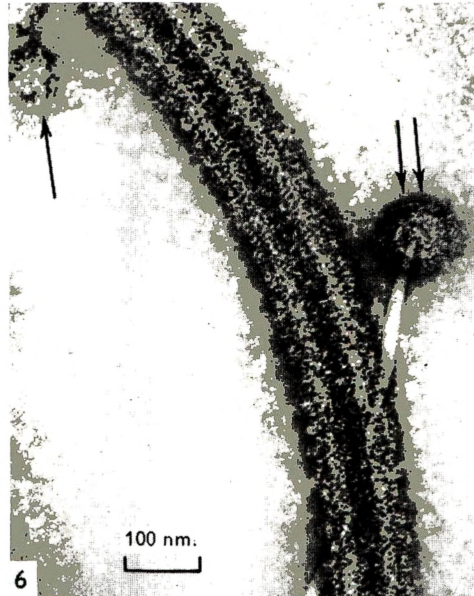

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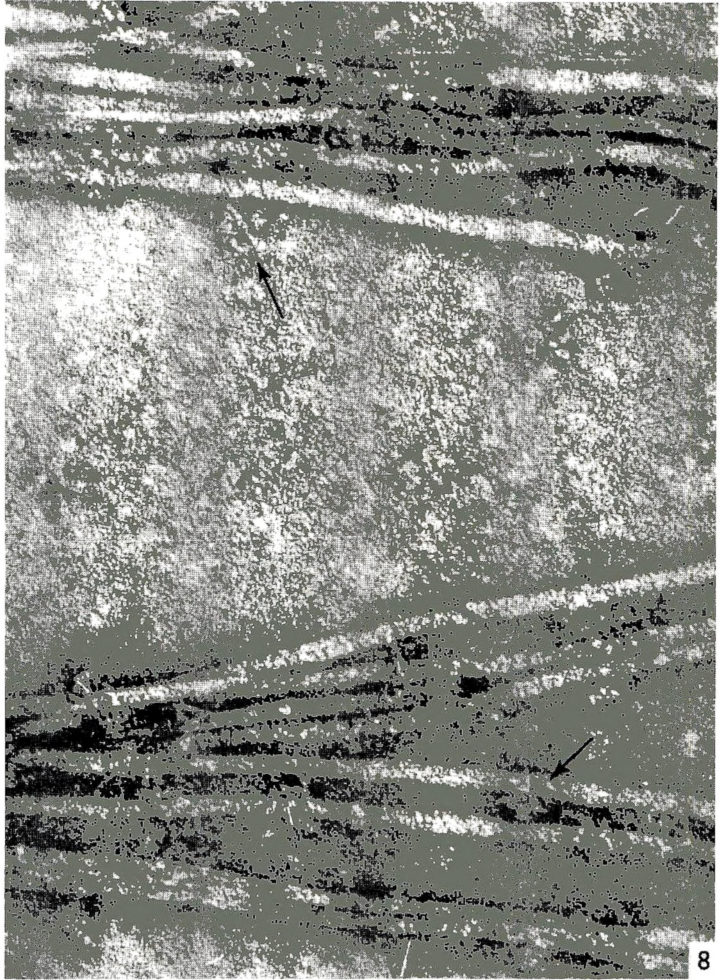

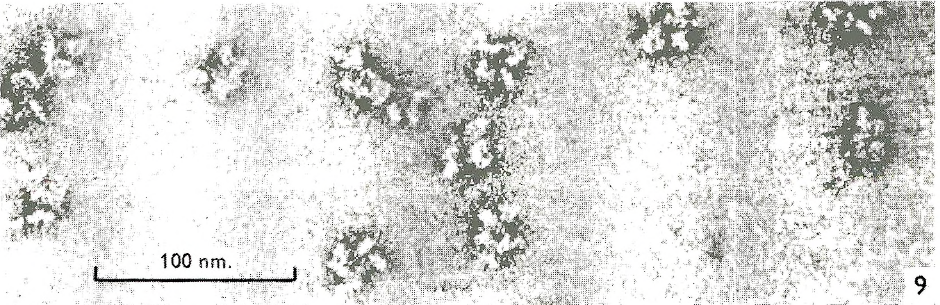

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Chandos House—2 Queen Anne Street—London W1M 9LE

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

THE

JOURNAL OF HYGIENE

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

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VOLUME LXVIII. 1970

CAMBRIDGE

AT THE UNIVERSITY PRESS

1970

PUBLISHED BY
THE SYNDICS OF THE CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W.1
American Branch: 32 East 57th Street, New York, N.Y. 10022

Printed in Great Britain at the University Printing House, Cambridge

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Printed in Great Britain at the University Printing House, Cambridge