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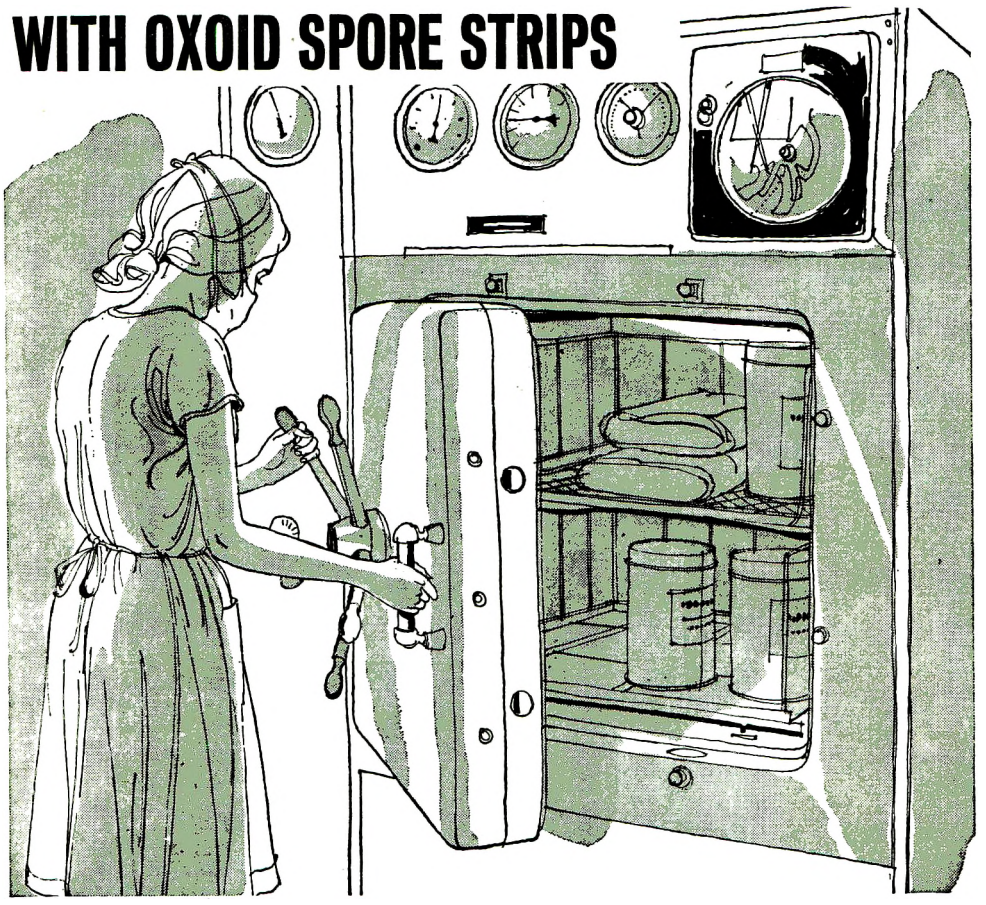

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Corrigendum

R. E. O. WILLIAMS, *J. Hyg., Camb.* (1967). **65**, 207–17.

On page 209, paragraph 4 (*Numbers of* Staphylococcus aureus *in the air*), in lines 5 and 6, for c.f.u./ft.^{2}/24 hr. *read* c.f.u./0·2. ft.^{2}/24 hr.

On page 210, in Fig. 2, in the left-hand scale, for c.f.u./ft.^{2}/24 hr. *read* c.f.u./0·2 ft.^{2}/24 hr.; the figures in the right-hand scale should *read*, from top to bottom, 0·05, 0·025, 0·005.

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A comparison of RK13, vervet monkey kidney and patas monkey kidney cell cultures for the isolation of rubella virus

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(*Received* 3 *February* 1967)

INTRODUCTION

Clinicians have become increasingly concerned with the public health hazard of rubella because of the relationship between maternal rubella in early pregnancy and subsequent congenital malformation (Gregg, 1941). Laboratory techniques are now available to assist clinicians in their diagnosis. Studies on the immunological status of patients are of limited use, but direct demonstration of excretion of virus is particularly valuable in the investigation of maternal rubella and newborn infants with the rubella syndrome (Plotkin, 1964).

Different workers favour different methods for virus isolation. The interference technique employing primary vervet monkey kidney cultures (Parkman, Buescher & Artenstein, 1962) is generally used for routine purposes in the U.S.A., where such cultures are readily available commercially. In Britain, where these cultures are more difficult to obtain, most workers employ a continuous rabbit kidney cell culture line, RK 13 (Beale, Christofinis & Furminger, 1963), the presence of rubella virus (RV) being detected by the appearance of a characteristic cytopathic effect (McCarthy, Taylor-Robinson & Pillinger, 1963). Patas monkey kidney cell cultures have been shown to support the growth of RV (M. Butler, personal communication), but studies on their use for virus isolation from clinical specimens have not been reported.

This paper presents the results of a comparison of the sensitivity and reliability of different cell cultures for the isolation of RV from clinical specimens. These were tested simultaneously under identical conditions.

MATERIALS AND METHODS

Tissue culture

We compared RK 13, primary vervet monkey kidney (VMK) and patas monkey kidney (PMK) cell cultures in parallel. A limited number of specimens were also tested on a continuous line of rabbit kidney, LLC-RK 1 (Hull & Butorac, 1966). Although this line has recently been shown to be sensitive to RV and its cytopathic effect (CPE) is easy to detect, its use for RV isolations from clinical specimens has not been reported.

Primary VMK and PMK cell suspensions ready for dispensing into tubes were

supplied by the M.R.C. Hampstead, and Wellcome Research Laboratories respectively. These cell cultures were generally confluent in 5–6 days. The RK 13 cell cultures were subcultured weekly, using a trypsin (0·125 %)/versene (0·05 %) mixture. Confluent cell sheets were obtained within 2–3 days. The trypsin/versene mixture was also used for subculturing the LLC–RK 1 cell cultures. These cell cultures required at least 7 days and 1–2 changes of medium before becoming confluent.

Table 1

Tissue culture system	Approximate no. cells inoculated per tube	Growth medium	Maintenance medium
VMK	15,000–20,000 clumps in 1 ml. amounts	Hanks's LaH Calf serum 5 % Glucose 1 % Sodium bicarbonate 0·088 % SV 5 antiserum 0·2 %	Earle's LaH Foetal calf serum 2 % Sodium bicarbonate 0·2 % SV 5 antiserum 0·2 %
PMK	125,000 cells in 1 ml. amounts	Earle's LaH Calf serum 2·5 % Sodium bicarbonate 0·1 % SV 5 antiserum 0·2 %	As for VMK
RK 13	100,000 cells in 0·5 ml. amounts	Medium 199 Calf serum (Flow Laboratories) 5 % Sodium bicarbonate 0·1 %	Medium 199 Inactivated rabbit serum 4 % Sodium bicarbonate 0·132 %
LLC-RK_1	100,000 cells in 1 ml. amounts	Medium 199 Calf serum 5 % Foetal calf serum 5 % Sodium bicarbonate 0·1 %	Medium 199 Foetal calf serum 2 % Sodium bicarbonate 0·154 %

Antibiotics, penicillin 200 units/ml. streptomycin 200 μg/ml. were included in all media.

The media required for growth and maintenance of the four types of cell culture are shown in Table 1. All cultures were incubated in stationary racks at 36° C. until ready for use.

The addition of amphotericin B (1·6 μg/ml.) to RK 13 cultures occasionally produced vacuolation, and its use was therefore unsatisfactory. Mycostatin (30 units/ml.) provided a satisfactory alternative antifungal agent. M & B 938 has also been found to be a satisfactory alternative (M. Butler, personal communication).

Initially, 3 lines of continuous vervet monkey kidney cell cultures which were reported suitable for the recovery of RV were also included in the investigation; GLV 3 (Christofinis, personal communication), BSC-1 (Parkman, Buescher *et al.* 1964), and GMK-AH 1 (Günalp, 1965). Although these lines supported the growth of RV, we found them insensitive to small amounts of virus such as might be obtained in clinical specimens. These continuous vervet cultures were therefore excluded from the investigation.

Specimens tested

Pharyngeal swabs were obtained from thirteen patients with rubella at St Thomas's Hospital. Two swabs were simultaneously obtained from each patient and immediately immersed in a bottle containing 4–5 ml. Hanks's BSS containing bovine plasma albumen 0·5 %, penicillin 500 units/ml., streptomycin 500 μg/ml., amphotericin B 8 μg/ml. Two swabs were obtained from each patient in order to inoculate as much virus as possible on to the cell sheet. In addition, specimens consisting of early passage tissue culture fluid from either RK 13 or VMK cell cultures were obtained from other laboratories. All material was 'snap' frozen and stored at $-70°$ C. until tested.

Inoculation of specimens

Whenever possible, all three tissue culture systems were inoculated in parallel with 0·1 ml. of the specimen undiluted and at 10^{-1} and 10^{-2} dilutions. Three tubes per dilution were used for RK 13 and four tubes per dilution for VMK and PMK; all specimens were adsorbed for 1 hr. at room temperature. Better adsorption of virus was found to occur if specimens were inoculated directly onto the cell sheet rather than onto cell cultures on maintenance medium. The process appeared to be more effective at room temperature than at 37° C., at which temperature some inactivation of virus may have taken place. All tubes were thereafter rolled at 36° C. Medium was changed on the RK 13 cultures after 18–24 hr. incubation in order to eliminate some of the swab or cellular material found to cause non-specific effects.

Control titrations

A control titration of a pool of RV (Judith) was included with each test, virus being inoculated in parallel in each cell culture system at virus dilution 10^{-1} to 10^{-7}. Virus titres were estimated according to the method described by Reed & Muench (1938).

Identification of virus

VMK and PMK cell cultures were challenged on the 10th day, and checked for the presence of interference 3 days later. Echovirus type 11 (100–1000 TCD 50/ml.) was used to challenge VMK cell cultures. Since echovirus 11 produces a poor CPE in PMK cell cultures, a bovine enterovirus, M 6 (100–1000 TCD 50/ml.), was used to challenge these cell cultures (M. Butler, personal communication).

RK 13 and LLC-RK 1 cell cultures were examined daily for 10 days. If CPE occurred following primary inoculation, cells and fluid were passed into fresh cultures to confirm the presence of CPE, since non-specific effects were invariably encountered when original specimens were inoculated. These effects may be confused with CPE. In all cell culture systems, two further passages following primary inoculation were carried out before specimens were declared negative.

Neutralization tests to confirm the identity of viruses were performed, employing sera from rabbits that had received six intravenous injections at weekly intervals of RV (Judith).

Serum neutralization tests

Neutralization tests on sera from seven patients were carried out simultaneously in RK 13 and PMK cell cultures. RV was used at 100 TCD 50 in RK 13, and 50 InD 50 (50 % interference dose) in PMK. PMK cell cultures were challenged on the tenth day after inoculation. Sera were not inactivated. The serum-virus mixtures were incubated at 37° C. before inoculation (Dudgeon, Butler & Plotkin, 1964; Parkman, Mundon, McCown & Buescher, 1964). Serum titres were estimated by the method of Reed & Muench (1938).

RESULTS

Sensitivity

The number of isolations from throat swab fluid, together with the number of passes and time taken to give an unequivocal result are shown in Table 2. Although CPE could be detected in RK 13 cell cultures as early as 3–4 days after inoculation of specimens, a passage in fresh cultures was always necessary in order to eliminate non-specific effects. Despite this, RV was detected both more quickly and more frequently in RK 13 than in VMK or PMK cell cultures. Furthermore, titration of original throat swab fluid revealed a titre of 10 or greater, in five of thirteen specimens tested in RK 13, but in only one of the twelve tested in both VMK and PMK cell cultures. Although VMK cultures appeared more sensitive overall than PMK cell cultures, these two cell culture systems were almost equally sensitive on primary inoculation.

Table 2. *Efficiency of isolation*

	cell system		
	RK 13	VMK	PMK
Number of isolations on primary inoculation	10	5	6
Number of isolations on first pass	2	5	1
Number of isolations on second pass	—	1	2
Total number of isolations/number tested	12/13	11/12	9/12
Mean time in days required to give an unequivocal result	10*	21	23

* This includes time to confirm CPE.

In order to determine which culture system was most effective in detecting small amounts of virus, a number of specimens known to contain low titres, including harvests from organ culture experiments in progress, were titrated in parallel. Table 3 shows that the highest titres and highest isolation rates were obtained in RK 13. Although RK 13 cell cultures were the most sensitive in the above tests, virus that had been originally isolated in VMK in other laboratories, when titrated in parallel gave higher titres in VMK and PMK than in RK 13 (Table 4). This difference was, however, not observed in the high passage strain, R 12 and the attenuated vaccine strain, HPV 77 (Parkman, Meyer, Kirschstein & Hopps, 1966).

A series of specimens, including some clinical specimens previously found to be negative, were passed in parallel in LLC-RK 1 and RK 13. Higher titres were

consistently obtained in LLC-RK 1. In addition three specimens which previously yielded no virus in RK 13 were found to be positive in LLC-RK 1 (Table 5).

Reliability

Fig. 1 shows that the titre of our standard pool of virus varied less in RK 13 than in either VMK or PMK cell cultures. It was found that poor titres of standard

Table 3. *Ability to detect small amounts of virus*

Specimens	Cell system: Titre of virus (log_{10} TCD 50 or InD 50/0·1 ml.) shown where applicable		
	RK 13	VMK	PMK
RK 13*a*	2 passes	—	—
RK 13*f*	1 pass	—	1 pass
RK 13*g*	1 pass	—	2 passes
RK 13*i*	2 passes	—	—
O.C. 1 30/4	1·25	1·25	N.T.
O.C. 1 2/5	< 1·00	—	N.T.
O.C. 1. 4/5	1·30	0·50	N.T.
O.C. 1. 6/5	< 1·00	0·50	N.T.
O.C. 1. 7/5	< 1·00	—	—
O.C. 3. 16/5	1·50	1·00	1·50
O.C. 3. 20/5	1·00	N.T.	1 pass
TL P1824	1·00	—	1 pass
Mean number of days required to give virus titre	6·50	13	13
Total no. positive/no. tested.	12/12	4/11	5/8

When no virus was detected on primary inoculation, the number of passages required to detect virus are shown.

N.T. = not tested

O.C. = organ culture material (human embryonic trachea and nose).

RK 13*a*—*i* = specimens containing small amounts of RK 13 grown virus.

Table 4. *Comparative titres of passage material*

Specimen	Details	Pass no.	Titre (log_{10}TCD 50 or InD50/0·1 ml.) RK 13	VMK	PMK
	A. *Initial passages in VMK or PMK*				
HPV77	Attenuated RV Vaccine strain	79	2·50	2·75	2·75
R 12	High Pass. Toronto	25	2·70	2·75	2·75
TL	T/S. Infants. Rubella syndrome	3	1·30	2·00	1·75
DM		1	< 1·00	1·25	2·20
Ca		2	1·00	2·25	> 2·50
Gi		2	1·75	> 2·50	> 2·50
Hi		2	1·50	2·20	2·20
Si		2	1·75	2·00	2·50
	B. *Initial passages in RK* 13				
AL T/S	Infants. Rubella syndrome	1	> 2·50	1·50	2·00
AL CSF		1	> 2·50	> 2·50	> 2·50
BH T/S		1	4·75	4·50	1·50
HM T/S		5	2·50	2·75	1·00

Table 5. *Comparison of RV isolations in RK* 13 *and LLC-RK* 1

Specimen	Details	Cell culture system Titre of virus (log_{10}TCD 50/0·1 ml.) shown where applicable	
		LLC-RKI	RK 13
RV 5	Standard strain (Judith)	5·00	3·50
HPV 77	Attenuated RV vaccine strain	3·50	2·50
HS	T/S. Nurse. Rubella	1·50	1·00
DC*	T/S. Doctor. Rubella	+	+
SP	T/S. Nurse. Rubella	> 1·50	< 1·00
OC 13 15/5	Organ culture (H. embryonic trachea)	1·00	1 pass
OC 3 16/5	Organ culture (H. embryonic trachea)	> 1·50	0·75
BW*	T/S. Infant. Rubella syndrome	+	−
ST*	T/S. Child. Rubella	+	1 pass
YC*	T/S. Child. Rubella	+	−
AH*	T/S. Child. Rubella	+	−
ST	T/S. Child. Rubella	−	−
DM	T/S. Infant. Rubella syndrome	−	−
J.G. (1)	T/S. Infant. Rubella syndrome	−	−
J.G. (2)	T/S. Infant. Rubella syndrome	−	−
No. Positive		11/15	8/15

When no virus was detected on primary inoculation the number of passages required to detect virus are shown.

* Specimens which were not titrated.

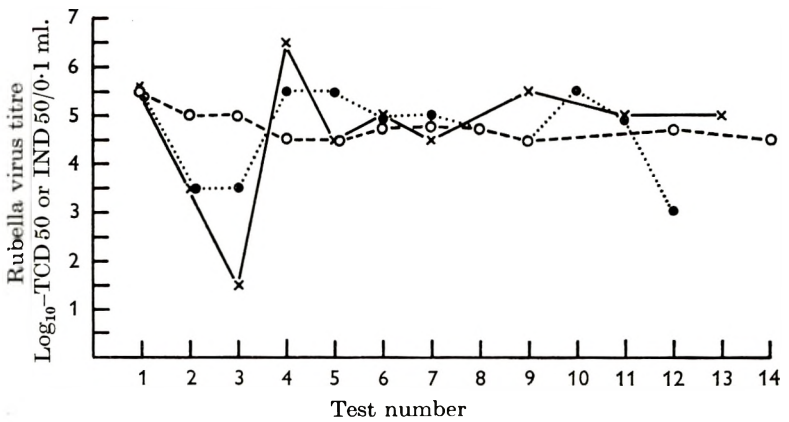

Fig. 1. Serial titrations of RV (Judith) in RK 13 (○—○) VMK (×—×) and PMK (●...●) cell cultures.

virus often coincided with poor cell sheets. However, the presence of simian syncytial and vacuolating agents did not usually cause widespread degeneration of the cell sheets or affect virus titre. The number of successful tests, with reasons for the failure of the others, are shown in Table 6. RK 13 cultures were usually maintained for two weeks without difficulty.

Serum neutralization tests

Results of serum neutralization tests with clinical details of patients studied are shown in Table 7. It can be seen that similar antibody titres were obtained in each system. Results were obtained by the 4th day in RK 13, but not until the 12–13th day in PMK cell cultures.

Table 6. *Condition of cultures*

	RK 13	V MK	PMK
No. satisfactory tests/no. tests performed	8/12	7/11	5/12
Difficulties encountered:			
Vacuolation	4	—	—
Failure to maintain	—	4	5
Primary MK cells not available, secondaries used	—	—	2

Table 7

Serum specimen	Details	Cell culture systems	
		RK 13	PMK
S.C.	Convalescent serum. Rubella	48*	48
J.G.	6-month-old infant. Rubella syndrome	48	48
M.G.	Mother of J.G.	48	48
B.Gr.	3-year-old child. Bilateral perceptive deafness	64	48
M.Gr.	Mother of B.Gr.	8	12
R.M.	Convalescent serum. Rubella	24	24
M.B.	Convalescent serum. Rubella	48	48

* Reciprocal antibody titre.

DISCUSSION

RK 13 cell cultures were seen to be the most sensitive for RV isolation, as shown by the higher isolation rates and greater capacity for detecting small amounts of virus. They were also the most reliable, as was shown by the comparative lack of variation in titres of the standard pool of RV. Being a continuous cell line, RK 13 cultures can be prepared whenever required; they also provided a result in a shorter time, a mean time of 9 days in this series, compared with 22 days by the interference technique.

However, the presence of RV in VMK and PMK cell cultures can be detected by the absence of CPE after challenge with echo 11 or M 6 more easily than by the CPE caused by RV in RK 13 cell cultures. This is of considerable advantage to inexperienced workers especially if large numbers of tubes are being used. PMK cells are more readily available commercially in this country than VMK, as patas monkeys are frequently used for vaccine production. Although secondary cells can be more readily transported than primary cells, no results have been reported for the comparative sensitivity of primary and secondary PMK and VMK cell cultures for RV isolation.

More specimens were found positive and even higher titres of virus were obtained in LLC-RK 1 than in RK 13. If sufficient virus was present it brought about complete destruction of the cell sheet in 5–7 days, this being more easily detected than the diffuse CPE obtained in RK 13. However, an insufficient number of specimens were compared in parallel in LLC-RK 1 and RK 13 to draw firm conclusions.

Neutralization tests on patients' sera showed comparable antibody titres. The RK 13 cell system (Dudgeon *et al.* 1964) is used as the method of choice in this laboratory, as cell cultures can be prepared whenever required and a result is usually obtained 8 days earlier than by the interference technique.

This paper gives the results of a necessarily limited series of experiments. The shortage of suitable clinical material and the difficulties encountered in obtaining a regular supply of PMK and VMK cell cultures made a larger series impracticable. Nevertheless, even this small series demonstrated that RV isolation in RK 13 and LLC-RK 1 cell cultures was superior to monkey kidney cultures in both sensitivity and speed. However, good cell sheets are essential and experience in the evaluation of changes in these cultures is necessary. Because variations in the quality of cell cultures is frequently encountered and since clinicians, particularly in the assessment of rubella during pregnancy, require an accurate diagnosis as quickly as possible, it is advisable to employ two cell culture systems in parallel, e.g. RK 13 and either PMK or VMK. It is useful to have a reserve of RK 13 cells kept frozen by means of liquid nitrogen (Nagington & Greaves, 1962). This can be reconstituted whenever difficulties are encountered, as it may take some weeks to adapt a line imported from another laboratory.

SUMMARY

RK 13 and primary PMK and VMK cell cultures were compared for the isolation of RV by means of the simultaneous inoculation of original specimens and early passage material. RK 13 was found to be the most sensitive and reliable and provided a result for both isolation and neutralization in the shortest time. As the interpretation of CPE and the propagation of these cultures is sometimes difficult, the simultaneous use of a second system in which RV is easy to identify, e.g. VMK or PMK cell cultures, is recommended. Both PMK cell cultures and LLC-RK 1 were suitable for isolating RV from clinical specimens. Preliminary studies with LLC-RK 1 indicate that it may provide an even more sensitive alternative to RK 13, but further studies employing clinical material require to be carried out before firm conclusions can be reached.

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Factors affecting the production of foot-and-mouth disease virus in deep suspension cultures of BHK21 Clone 13 cells

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It was shown by Capstick, Telling, Chapman & Stewart (1962) that BHK 21 Clone 13 cells would multiply in suspension culture and remain susceptible to foot-and-mouth disease (FMD) virus. Recently Telling & Elsworth (1965) and Telling & Stone (1964) have demonstrated that these cells can be grown in a 30 l. vessel equipped with automatic pH and temperature control apparatus, thus opening the possibility of producing FMD inactivated vaccines on a commercial scale.

The experiments described here were undertaken to determine the optimum conditions for viral infection in deep suspension culture to release maximum FMD viral antigen for use in inactivated vaccines.

MATERIALS AND METHODS

Cells

BHK 21 Clone 13 cells were used after passage for between 100 and 150 days in suspension culture. They were then grown for 3 days in 2 l. glass vessels as described by Telling & Elsworth (1965). After a growth period of 3 days the cells were infected with virus. The exhausted medium was removed by centrifugation and unless otherwise stated the cells were resuspended for virus infection in Eagle's medium with 10% tryptose phosphate broth (T.P.B.) and 5% horse serum. Antibiotics were incorporated at final concentrations of penicillin and streptomycin 100 units, neomycin 70 units, polymixin 20 units and mycostatin 25 units/ml. The bicarbonate content of the complete medium was adjusted to a final concentration of 3·0 g./l. Antifoam (Emulsion RD)* was added at 0·02%. The cells used in these experiments were at least 90% viable at the time of infection.

Virus strains

All virus used originated from infected cattle epithelium. The virus was passaged in BHK monolayers, and the supernatant fluid frozen as stock seed virus in 60 ml. volumes at $-70°$ C. One stock of seed virus was used for a complete series of experiments involving one variable. No deliberate attempt was made to adapt

* Midland Silicones Ltd., London.

the viruses to BHK cells, and only sufficient passages were carried out to provide enough virus for stock seed. The viruses used were:

Type O	Strain Israel 1/63	6th BHK passage
Type A	Strain A 119	7th BHK passage
Type A	Strain Iraq 24/64	7th BHK passage.

Virus assays

Infective virus titres were assayed by the plaque technique on 2-day monolayers of BHK 21 Clone 13 cells. Plaques were counted 2 days after infection.

Complement-fixing antigen titres were estimated using Arklone P extracted supernatant culture fluids as described by Brown & Cartwright (1960). In preliminary experiments it was determined that the use of Arklone P in the presence of serum does not remove the 7 mμ virus component, but it does, however, remove most of the anticomplementary activity present in some cultures. Culture supernatant fluids were extracted by manual shaking three times with an equal volume of Arklone P. Complement-fixing antigen titres were determined by a modification of the method of Bradish, Jowett & Kirkham (1964). Two-fold dilutions of antigen, one 70% dose of complement and the optimal antiserum dilution were used in the test. After fixation for $1\frac{1}{2}$ hr. at 37° C. and addition of the haemolytic indicator system, haemolysis was allowed to proceed for 1 hr. at 37° C. The r.b.c. were then deposited by centrifugation and the degree of haemolysis estimated in an absorptiometer. The antigen titre of a sample was expressed as complement fixing units (cfu) and was the reciprocal of the antigen dilution causing 30% reduction in haemolysis compared with the controls.

In our hands this test has a 'between test' standard error of 14% ($P = 0{\cdot}05$) and a 'within test' standard error of 5% ($P = 0{\cdot}05$).

Culture vessels

Two identical 800 ml. culture vessels were constructed. These vessels were of similar proportions and construction to the bacterial culture vessel described by Elsworth, Capel & Telling (1958) but without baffles.

The vessels were equipped with automatic pH and temperature recording controlling apparatus as described by Telling & Stone (1964), and Telling & Elsworth (1965). The pH was controlled at the desired value $\pm$ 0·05 pH unit and the temperature at the desired value $\pm$ 0·5° C. The vessels were sterilized *in situ* by direct steam injection at 4 lb./in.^{2} for 4 hr. Effluent air from virus infected vessels was sterilized by heating in a tube furnace at 400° C.

Virus–cell cultures

Both vessels were used for virus–cell experiments. One vessel was used to determine the effect of a variable and the other maintained as a control. The medium and cells were prepared in bulk in glass containers and a measured volume transferred to the vessels under slight positive pressure of filtered air. The temperature, pH control and stirring mechanisms were then put into operation and

when the desired pH and temperature values were reached the culture was infected with virus. The thawed stock seed virus was added as 10 % of the culture volume. Where necessary the stock seed was diluted in medium to give the required infective titre in the appropriate volume.

Samples of the cultures were removed at frequent intervals after infection and the cell concentration was determined after trypan blue staining. The cells were then removed from the sample by centrifugation and the supernatant stored at 4° C. to await infectivity and complement-fixing titrations.

In the later stages of an infected culture it was difficult to obtain accurate estimates of dead cell numbers as the cell count was obscured by increasing amounts of cell debris. It was found that a useful estimate of the progress of viral infection could be obtained by counting only the surviving viable cells and expressing these as a percentage of the starting viable cell concentration.

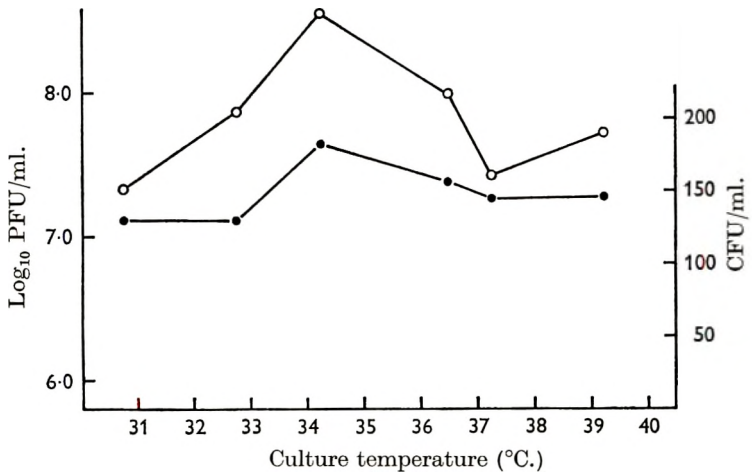

Fig. 1. The effect of culture temperatures on peak extracellular FMD A/Iraq virus titres. ○—○, Open circles pfu/ml. ●—●, Closed circles cfu/ml.

RESULTS

Effect of temperature

The effect of temperature on peak extracellular A/Iraq virus titres is shown in Fig. 1. At least two observations were made for each point. Virus titres declined on either side of 34·25° C., but estimating from the shape of the graphs the optimal temperature is probably nearer to 35° C.

The culture temperature had a profound effect upon cell survival following infection. This is shown in Table 1 in terms of the 30 % cell survival time at 34·25° C. At temperatures below 34·25° C. cell survival increased markedly, apparently due to reduced virus replication. This was reflected by lowered peak virus titres.

Table 1. *Effect of culture temperature on* 30 % *cell survival time of cells infected with A/Iraq FMD virus*

Culture temperature	30 % Cell survival time
(°C.)	(hr.)
30·75	70·5
32·75	51·6
34·25	25·5
36·5	22·2
37·25	16·5
39·25	26·2

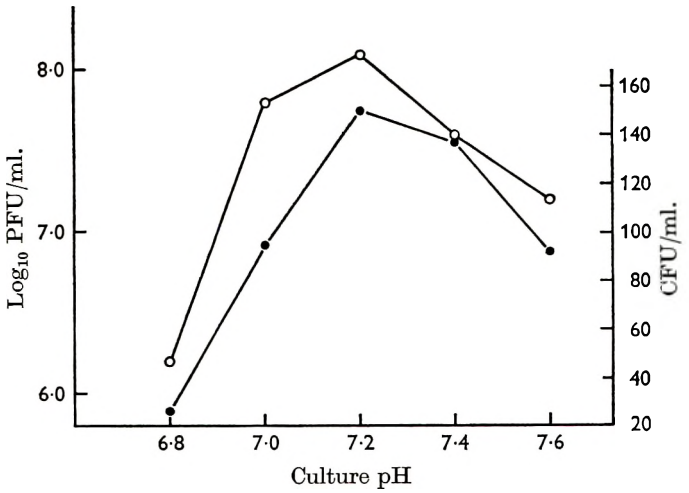

Fig. 2. The effect of culture pH on peak extracellular FMD A 119 virus titres. ○—○, Open circles pfu/ml. ●—●, Closed circles cfu/ml.

Effect of culture pH

The peak extracellular virus titres were examined over the pH range 6·8–7·6 at 0·2 pH intervals. The results using A 119 virus are shown in Fig. 2. Maximum extracellular virus titres were reached at pH 7·2 and declined above and below this value. The 30 % cell survival time decreased from 46 hr. at pH 6·8 to approximately 22 hr. at pH 7·2 and above.

The pattern of infective extracellular virus release during the period of an infected culture at pH 7·2 and 7·6 is shown in Fig. 3. Although at high pH levels cell infection and virus release appeared to take place normally during the first 4 hr. of culture, virus reproduction was much reduced in the later stages.

Cell concentration

In this series of experiments 'O' Israel seed virus was appropriately diluted before infection of the culture to maintain an approximate input virus/cell ratio of one plaque-forming unit (pfu) per 30 cells. The effect of cell concentration on the extracellular virus titres and on number of pfu per cell and cfu per $10^{6\cdot0}$ cells is shown in Table 2, for the range $1 \times 10^{6\cdot0}$ to $9\cdot6 \times 10^{6\cdot0}$ cells/ml. Infective antigen

titres per ml. showed no increase over this range. Complement-fixing antigen titres per ml. increased as the cell concentration increased, but not in direct proportion. As the cell concentration was increased the yield per cell either of complement fixing or infective antigen decreased.

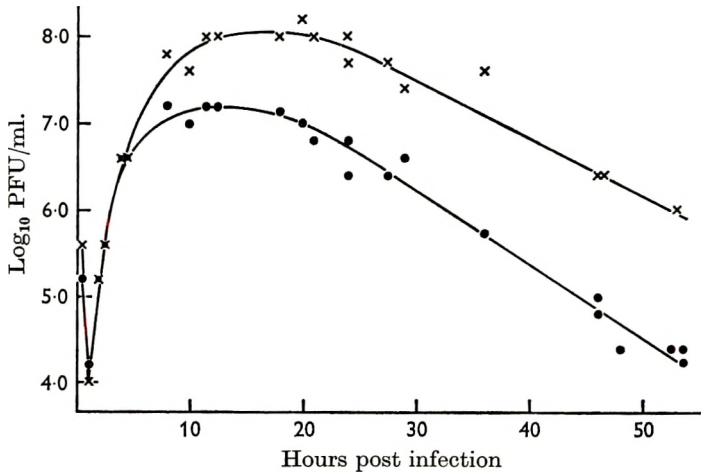

Fig. 3. The extracellular infective virus released from BHK 21 Clone 13 cells during cultures held at pH 7·2 and pH 7·6. × — ×, Crosses pH 7·2. ● — ●, Closed circles pH 7·6.

Table 2. *Effect of cell concentration on extracellular virus titres and antigen released per cell*

Cell concentration per ml. × $10^{6\cdot0}$	Infective antigen		Complement-fixing antigen	
	pfu./ml.* $\log_{10}$	pfu/cell*	cfu/ml.*	cfu/10^6 cells
1·1	7·8	56·5	79·4	72·5
1·9	8·0	53·4	93·6	50·5
2·5	7·9	31·9	75·3	30·6
3·0	7·7	15·2	124	41·0
4·6	8·2	35·5	155·5	33·8
7·5	8·0	12·6	196·5	26·1
8·0	7·9	9·2	198·0	24·7
9·6	7·8	7·0	N.D.	—

* Measured at the time of peak titre.
N.D. = not determined.

Multiplicity of infection

The volume of seed virus required to infect a culture is of considerable importance on a large scale and experiments to determine the effect of varying the input virus/cell ratio were carried out. During these experiments cell concentrations were kept in the range 2·4–3·2 × $10^{6\cdot0}$ cells/ml. The results expressed in yield of virus antigen per cell at the time of peak titre are shown in Table 3 for A/Iraq virus. The yield of complement-fixing antigen per cell was independent of the input virus/cell ratio. The infective yield per cell was significantly reduced at virus/cell ratios below 1:320.

The effect of serum

The inclusion of 5 % filtered horse serum in the medium results in increased infective and complement-fixing antigen titres. A typical experiment is shown in Table 4. In the culture in which serum was included there was an approximate threefold increase in complement-fixing antigen. We have been unable to demonstrate a similar effect following infection of monolayer cultures of BHK cells. The addition of ox serum produced similar results, but some serum batches were found to contain non-specific neutralizing activity which considerably reduced the antigen titre of some virus strains.

Table 3. *Effect of input virus/cell ratio on the virus released per cell*

Log no. of cells to one pfu	Infective units/ cell* (pfu)	Complement-fixing units* per $10^{6\cdot0}$ cells
0·59	51·5	57·1
1·12	51·6	42·8
2·50	52·1	36·6
3·56	26·8	67·4
3·66	36·8	45·2
4·75	21·1	44·1
5·32	36·1	49·8
5·88	18·6	46·9

* Measured at time of peak titre.

Table 4. *The effect of addition of 5 % horse serum to cultures infected with A 119 FMD virus*

Time post inoculation (hr.)	No serum		5 % horse serum	
	pfu/ml. $\log_{10}$	cfu/ml.	pfu/ml. $\log_{10}$	cfu/ml.
3	5·4	N.D.*	5·2	N.D.
20·5	7·25	25	7·81	N.D.
22·5	7·34	30	7·90	45
28	7·29	39	7·88	100
45	6·48	41·5	7·20	108
52	N.D.	49·0	N.D.	124
69	N.D.	44	N.D.	150

* N.D. = not determined.

DISCUSSION

The results obtained here must be interpreted in the light of the desired end-product, and the processes following the virus culture. Thus, if only high titres of infective virus are required the culture can be terminated at a much earlier stage than if maximum antigen (as expressed by complement-fixing activity) is the required end-product. With all the virus strains used here the time of peak complement-fixing activity occurred approximately 24 hr. or longer after the time of peak infective virus titre.

Temperature and pH control during virus infection were found to be necessary if maximum titres are required. However, in the case of pH control, it is not known

whether pH was the only factor contributing to the observed variations in peak titre. For example, the method of pH control used calls for sparged air beneath the impeller if the pH shifts to the acid side of the controlled value. As cultures continually produce acid during the first 48 hr. of infection, those cultures controlled at a high pH value receive more air, and therefore more oxygen than cultures controlled at low pH values. It is thus possible that dissolved oxygen or the effect of sparging on the cells may have been contributing factors to the results. The time of peak infectivity in the cultures was not affected by changes in the input virus/cell ratio over the range described in Table 3. During this series of experiments the maximum infectivity titre occurred at 23 ± 3 hr. after virus infection.

Assessment of the best cell concentration for use during vaccine production is difficult as the system becomes increasingly inefficient with increasing cell concentrations. For inactivated FMD vaccines a high complement-fixing antigen titre is required (Brown & Newman, 1963). However, it is difficult to fix a minimum acceptable CF antigen titre applicable to all strains of FMD virus, as the amount of complement-fixing antigen produced varies with the virus strain used. Similarly, vaccines prepared from a range of virus strains have varying immunogenic potential when measured *in vitro* (authors–unpublished information). Each virus strain and the level of CF activity required must be assessed separately, bearing in mind the vaccine dose rate, composition, etc. We have found that vaccines containing 1 ml. of antigen prepared from cultures with cell concentration of $2{\cdot}0–2{\cdot}5 \times 10^{6{\cdot}0}$ cells/ml. confer satisfactory protective levels in cattle; but this can only be used as a general guide.

If an efficient virus concentration procedure was envisaged in the later stages of production, it would be more productive to infect cells at a concentration of $1 \times 10^{6{\cdot}0}$/ml.

In the series of experiments dealing with input virus/cell ratios, peak infective titres remained the same, and occurred at the same time with input virus/cell ratios ranging from 1:1 to 1:320. This is of considerable interest from a large-scale production viewpoint, as theoretically 1 l. of monolayer seed virus (approximate titre $10^{7{\cdot}0}$ pfu/ml.) could be used to seed a 400 l. culture vessel without a reduction in the titre of peak virus. It is of interest that Telling, Radlett & Mowat (1967), using the same cell system, found that Semliki Forest virus yield increased as the input virus decreased.

The use of serum in the medium during the infective process, while giving a valuable increase in antigen titre, may well be inconvenient in many cases where virus is produced for biochemical or immunochemical studies. Bovine serum frequently contains specific neutralizing activity in those countries where FMD is endemic and horse serum is not always available in quantity. However, in our opinion the increases in antigen titres are sufficient to warrant the inclusion of serum in the virus culture medium at present. The ideal solution would be the development of a strain of cells which was not serum-dependent during the cell growth and virus infection processes and further work is being carried out on these lines.

SUMMARY

For maximum utilization of deep cultures to produce FMD virus it was important to have adequate control of culture temperature and pH. Culture temperature should be controlled within the range 34·25°–35° C. and culture pH at 7·2. The culture system became less efficient as the cell concentration was increased from $1 \times 10^{6 \cdot 0}$ to $9 \times 10^{6 \cdot 0}$ cells/ml. A cell concentration of $2 \cdot 5 \times 10^{6 \cdot 0}$ cells/ml. represented a working compromise between efficiency and antigen titre/ml. for inactivated FMD vaccine production.

The input virus/cell ratio had no effect on the time or titre of peak virus yield in the range 1:1 to 1:320. This makes the production of seed virus from small numbers of monolayer cultures feasible and economical.

Virus yield was improved by the addition of 5% serum. It would be more satisfactory if a serum-free cell strain could be developed.

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Features of molluscum contagiosum in the north-east of Scotland and in Fijian village settlements

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INTRODUCTION

Molluscum contagiosum is a benign, human skin tumour of world-wide distribution whose epidemiological features have been briefly summarized by Overfield & Brody (1966). It is caused by a virus which morphologically resembles those of the pox group but which, hitherto, has not been grown in regular serial passage outside the human host. Consequently neutralizing antibodies have only been detected with difficulty and the pathogenesis of the condition is far from clear. Furthermore, the long and variable incubation period, the sporadic occurrence of the disease and its trivial nature and spontaneous cure have precluded a precise definition of its natural transmission between hosts. Histologically, the superficial localization of the tumour and the complete absence of host cellular reaction are as striking features as is the Henderson–Paterson inclusion body, which is the hallmark of the disease. To shed more light on this condition clinico-epidemiological studies are being carried out in two widely separated and racially distinct population groups: hospital out-patients and domiciliary patients in Aberdeen and the north-east of Scotland and Fijian village dwellers in the islands of Fiji in the south-west Pacific. This report presents preliminary findings of these studies.

MATERIALS AND METHODS

The populations studied

The Aberdeen surveys

During 1964 patients with clinically typical molluscum contagiosum were referred from the Dermatology Out-Patient Clinic of Aberdeen Royal Infirmary and by local general practitioners. Histories were recorded according to a standardized series of questions, clinical details were recorded and the central cores of lesions were expressed. Some lesions were further treated by phenol cauterization. The striking age and sex distribution revealed in this survey prompted a further inquiry into the hospital records of all cases of molluscum contagiosum attending the Skin Out-Patient Clinic during the years 1956–63. During an extension of this work in 1966, thirteen further cases were examined by a different observer.

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The Fiji surveys

In 1965 and 1966 two medical students from Aberdeen, during 3-month scholarships in Fiji sponsored by the Nuffield Foundation, undertook, as part of wider studies, small surveys of molluscum contagiosum in selected communities.

(i) In 1965 a survey of pre-school children was carried out in the low-cost housing settlement of Nabua, about $3\frac{1}{2}$ miles from the centre of Suva, the capital, on the island of Viti Levu. One-third of the population, of about 890, was housed in sub-standard dwellings and here it was common practice for the whole family, usually with about five children, to live in a single room. The remaining two-thirds of the population lived in concrete flats with a large bedroom, a small living room, a kitchen and a shower/lavatory unit. Half this part of the settlement was surveyed.

(ii) To provide more data on this age group (0–5 years), results pertaining to molluscum contagiosum were abstracted from a Report on a more comprehensive medico-social study carried out in 1959 at Naviti, an island in the Yasawa group, north-west of Viti Levu. On this island seven village communities, each of up to 200 people, live in buildings of wood and thatch or of wood and corrugated iron, arranged around a central clearing, in which children from all dwellings play together.

(iii) In 1966 a survey was carried out on 90 % (672 persons) of the entire population of Wailoku, an Anglican Mission Settlement near Suva. These people were predominantly descendants of Solomon Island indentured labourers who had married with local Fijians.

(iv) Concurrently, the patients who were noted to have molluscum contagiosum in 1965 ((i) above) were re-examined in 1966.

Laboratory confirmation of the clinical diagnosis

Although the lesions of molluscum contagiosum are so typical the following confirmatory laboratory tests were carried out. Electron microscopic examination by the negative staining technique on extracts of lesions from Aberdeen and Fiji showed large numbers of virus particles typical of the pox group (Plate 1A and B). In the absence of a convenient laboratory test for detecting infectivity of the molluscum virus, extracts were examined for biological activity by their capacity to inhibit plaque production by vaccinia virus in mouse embryo cell cultures (Postlethwaite, 1964). Extracts from Fijian lesions were as active in this respect as those from Aberdeen. Finally, a representative lesion from Fiji, transported in 50 % buffered glycerol to Aberdeen, was sectioned and stained by phloxine-tartrazine. Histological examination showed the characteristic appearance of molluscum contagiosum (Plate 1C).

RESULTS

The Aberdeen surveys

In spite of their characteristic appearance, the lesions of molluscum contagiosum were often confused by patients with those of verruca vulgaris. Moreover, of 167 doctors' referral letters on patients under 12 years seen at the Skin Out-Patient

Clinic, 90 gave the correct diagnosis, 63 diagnosed warts and 14 were simply descriptive.

In 1964 thirty-nine patients with typical molluscum contagiosum were seen. Their distribution by age and sex is shown in Table 1. Of the 35 aged 7 to 15, 33 gave a history of swimming at public baths within a short period before the development of molluscum lesions. Most were regular attenders. One of the two non-swimmers frequently used communal spray baths. As determined from a control group of children, questioned in 1966 in the same area and corrected for age and sex, the expected number of swimmers was 17·6 ($\chi^2 = 13{\cdot}2$, $P < 0{\cdot}01$).

Table 1. *Distribution by age and sex of patients with molluscum contagiosum in Aberdeen*

	Number of cases							
	1964		1956–63		1966		Totals	
Age in years	M	F	M	F	M	F	M	F
1–3	0	2	5	9	0	0	5	11
4–6	0	0	5	6	0	1	5	7
7–9	6	2	31	8	3	0	40	10
10–12	17	3	103	30	5	0	125	33
13–15	3	4	57	22	3	1	63	27
16–18	0	2	8	7	0	0	8	9
> 18	0	0	13	12	0	0	13	12
Totals	26	13	222	94	11	2	259	109

M = male; F = female.

Because of the high incidence of boys aged 10 to 12 years details of age and sex of all previous cases of molluscum contagiosum attending the Skin Out-Patient Department in the years 1956–63 were collected (Table 1). These figures were compared with those of the control sample already mentioned, obtained by asking 1848 schoolchildren aged 7 to 15 how many had attended swimming baths in the previous 6 months. Table 2 shows the age and sex distribution, with the percentage of swimmers, in this control group. No significant difference was found between the age distributions in the two groups ($\chi^2 = 0{\cdot}96$). A highly significant difference in the sex distribution ($\chi^2 = 32{\cdot}15$, $P < 0{\cdot}001$) was found, and clearly the incidence of molluscum contagiosum was much greater in males than females within this age group.

The previous medical histories of the 39 cases studied did not suggest any positive or negative correlation of common childhood illnesses with molluscum contagiosum. Ninety-two per cent had been successfully vaccinated against smallpox in infancy. This figure is not significantly different ($\chi^2 = 2{\cdot}53$) from the expected figure of 73% (M.O.H. Report for the City of Aberdeen, 1953).

Less than 25% of the patients knew of other cases of molluscum contagiosum. In no instance was another member of the same family or household known to be similarly infected. Sixty-six per cent of the patients attended the Skin Out-Patient

Department within 4 months of noticing a molluscum lesion. More than 25% waited 6 months or more. Two out of 39 claimed to have had lesions continuously for at least 1 year. One patient had had molluscum contagiosum previously; more than 3 months had elapsed between his apparent cure and the recurrence. A second case returned with new lesions 6 weeks after an apparently successful treatment. There was no marked seasonal incidence. Of 38 cases in whom it was possible to estimate the month of appearance of lesions, 11 began in Spring (March to May) and 9 in each of the other seasons. All patients were able to identify the site of the first apparent lesion although they were often uncertain about the

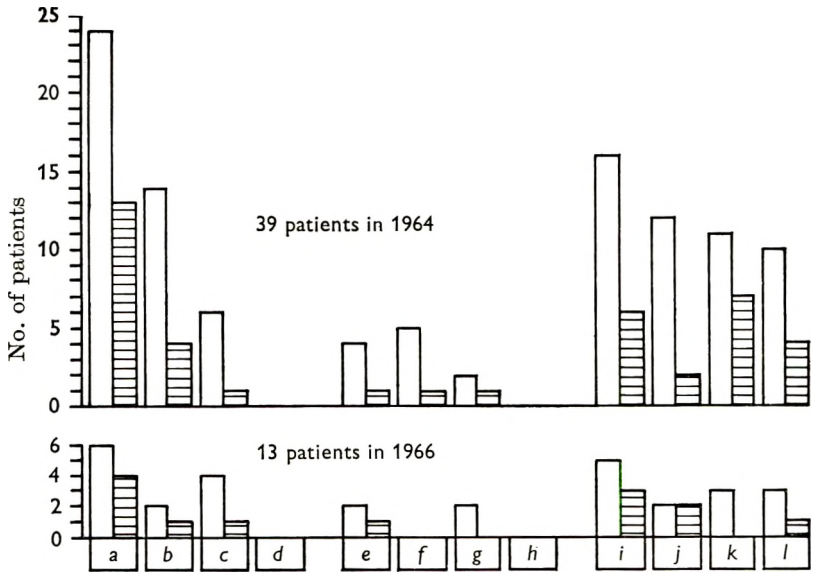


Fig. 1. Anatomical distribution of molluscum lesions seen in Aberdeen patients in 1964 and 1966. □, Lesions noted at examination; ▤, initial lesions.
a = axilla; *b* = shoulder and upper arm; c = fore-arm and elbow; *d* = hand; *e* = inguinal region; *f* = thigh; *g* = knee-leg; *h* = foot; *i* = chest; *j* = abdomen; *k* = face and neck; *l* = buttock and back.

initial lesion in a cluster. Figure 1 shows the anatomical distribution of initial lesions in the 39 cases studied, and also the number of patients presenting with lesions in different sites.

The number of lesions found on first examination ranged from 1 to 73 with a mean of 16. Of 35 cases with multiple lesions 20 showed a unilateral distribution. Most lesions were 1–3 mm. in diameter, and the largest recorded was 7 mm. A history of associated symptoms was specifically sought; 61% had local erythema, 58% pruritis, 55% bleeding, 39% secondary bacterial infection and 29% tenderness or pain.

Treatment was by expression of the central core and, in certain cases, cauterization of the base with phenol. At a 4-week follow-up of 26 patients, treatment was apparently successful in 9 out of 13 treated by expression alone and in 10 out of

13 treated by expression and cauterization. There was clearly no significant difference in cure rates at 4 weeks.

Relevant features of the 13 patients seen during 1966 are presented in Tables 1 and 3 and in Fig. 1. Seen by a different observer, they confirm, in essential details, those features noted in the 1964 survey.

Table 2. *Distribution by age and sex of children in control group, with percentage of swimmers* (1966)

	Males			Females		
Age in years	Sample	Swimmers	Swimmers (%)	Sample	Swimmers	Swimmers (%)
7–9	229	79	35	248	78	31
10–12	372	211	57	366	234	64
13–15	318	182	57	315	131	42
Totals	919	472	51	929	443	48

Table 3. *Features of molluscum contagiosum in* 13 *patients seen in Aberdeen during* 1966*

	Children
Swimmers	13
Vaccination history	10 (3 not known)
Season of onset:	
Spring	5
Summer	3
Autumn	2
Winter	3
Unilateral lesions	6
Family doctor's diagnosis:	
Molluscum contagiosum	7
Warts	6
Other	0
Itch/pain/bleeding/infected lesion	7
Contact or family history	1†
Time lesions present before seen at clinic	4·4 months (range 2–10)
Interval between initial and later lesions	Few days to 3 months
Number of lesions present on examination	15·2 (range 5–43)

* Age and sex distributions appear in Table 1 and the anatomical distribution of lesions in Fig. 1.

† A twin brother developed lesions 1 month after onset in index case.

The Fiji surveys

The age and sex incidence of molluscum contagiosum in the Fiji surveys are presented in Table 4. The 1966 figures show that whilst the sex incidence was equal for the population as a whole, 53 % of cases occurred between 1 and 5 years. This accounts for the slightly lower overall incidence (4·5 %) compared with the 7·2 and 6·2 % incidences noted in 1959 and 1965 respectively. During these years only pre-school children were studied, though questioning of mothers during 1965 elicited no history of molluscum contagiosum in older members of the family.

Table 4. *Distribution by age and sex of patients with molluscum contagiosum in Fiji*

Number of cases according to source of material

Age in Years	(a) 1959 report. Naviti Island. 0–5 years			(b) 1965 survey. Nabua. 0–5 years			(c) 1966 follow-up of 1965 survey. Nabua. New lesions			(d) 1966 survey. Wailoku. Whole population			(e) Totals (less (c))		
	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T
1	0	0	0	1	2	3	0	1	1	0	1	1	1	3	4
2	3	6	9	0	1	1	0	1	1	1	3	4	4	10	14
3	0	3	3	2	0	2	1	0	1	3	2	5	5	5	10
4	0	2	2	0	0	0	0	0	0	1	2	3	1	4	5
5	0	0	0	2	0	2	0	0	0	2	1	3	4	1	5
6	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0
7	.	.	.	.	.	.	.	.	.	2	0	2	2	0	2
8	.	.	.	.	.	.	.	.	.	0	1	1	0	1	1
9	.	.	.	.	.	.	.	.	.	2	0	2	2	0	2
10	.	.	.	.	.	.	.	.	.	1	0	1	1	0	1
11	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0
12	.	.	.	.	.	.	.	.	.	1	1	2	1	1	2
13	.	.	.	.	.	.	.	.	.	1	2	3	1	2	3
14	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0
15	.	.	.	.	.	.	.	.	.	0	1	1	0	1	1
> 15*	.	.	.	.	.	.	.	.	.	1	1	2	1	1	2
Totals	3	11	14	5	3	8	1	2	3	15	15	30	23	29	52
Total examined	96	84	180	69	60	129	.	.	.	326	346	672	491	490	981
% with M.C.	3·0	13	7·2	7·3	5·0	6·2	.	.	.	4·6	4·3	4·5	4·7	5·9	5·3

Note. In 1965, two other cases of molluscum contagiosum were seen, in the Yasawa group of islands: a male aged 3 and a female aged 1 year. * One female of 30 and a male of 32 years.

M = male; F = female; T = total.

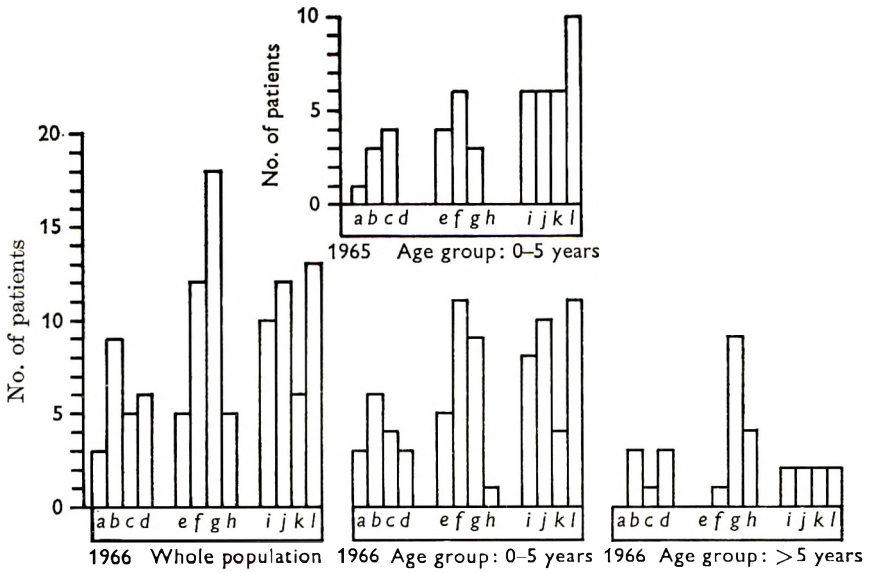


Fig. 2. Anatomical distribution of molluscum lesions in Fijian patients, as noted on examination. Letters *a* to *l* denote anatomical regions as in Fig. 1.

The anatomical distribution of lesions in the Fijian patients, as they presented on examination, is shown in Fig. 2, which also presents the data of the 1966 survey separately for the pre-school and older patients. The suggestion that fewer skin areas were affected in older patients was confirmed by direct scoring. For this purpose skin areas corresponding to those indicated in Fig. 2 were scored if they presented molluscum lesions, left and right limb areas being scored separately

Table 5. *The recurrence of molluscum lesions in the eight Fijian patients from Nabua as affected by surgical removal of lesions one year previously*

Patient		Number of lesions in 1965	Treatment in 1965	Number and site of new lesions in 1966
Sex	Age			
M	1	9	Nil	Nil
M	5	10	Nil	Nil
F	1	7	Nil	1 left elbow 1 right thigh 1 right buttock } 3
F	2	7	Nil	1 right buttock 1 right great toe } 2
F	1	27	3 lesions excised from chest and back	Nil
M	3	9	2 lesions excised from back and buttocks	Nil
M	5	22	2 lesions excised from neck and left elbow	Nil
M	3	10	2 lesions excised from abdomen	2 left buttock 1 right buttock 1 right thigh 1 right knee } 5

M = male; F = female.

whilst central skin regions (face and neck, chest, abdomen and back) were considered as single units. Genital lesions were included with existing inguinal lesions or were scored as inguinal in location if the latter site was not involved. On this basis, affected skin areas averaged 4·8 (range 1–11) for the age group 0–5 years and 2·1 (range 1–4) for the older age group. The corresponding figure for the younger children in the 1965 survey was 5·1.

In 1966 (30 cases) and 1965 (10 cases) the numbers of lesions ranged, respectively, from 1 to 71 (mean 15·2) and 7 to 27 (mean 15·9). Thirteen cases out of 30 in 1966 showed a unilateral distribution of lesions, including 3 cases with only 1, whilst in 1965 all lesions were distributed bilaterally.

The 8 cases from Nabua had a total of 101 lesions when examined in 1965. From 4 of these cases a total of 9 lesions was surgically excised, an operation which caused moderate bleeding. One year later none of the lesions was present, pale scars level with the skin surface being noted at the site of the previously recorded

lesions. Three children had new lesions in different sites. Table 5 shows no positive correlation between the occurrence of these lesions and the previous expectant or surgical treatment. The spontaneous resolution of molluscum lesions was very noticeable in Fijians. In both the 1965 and 1966 surveys, follow-up visits 5–7

Table 6. *Household incidence of molluscum contagiosum in the Fiji surveys*

	Source of data			
	1959 report. Naviti Island. 0–5 years	1965 survey. Nabua. 0–5 years	1966 survey. Wailoku. Whole population	Totals
Number of households examined	165	118	113	396
Number of households with one case only	8*	4*	18	30
Number of households with more than one case	3*	2*	5	10
Total number of households with molluscum contagiosum	11*	6*	23	40
Households with more than one case				

Household no.	Sex	Age	Sex	Age	Sex	Age
1	F	3	M	5	F	3
	F	2	M	3	F	2
2	F	2	F	1 } twins	M	13
	M	2	M	1 }	F	2
3	F	2	.	.	M	12
	M	2	.	.	F	8
4	.	.	.	.	M	7
	.	.	.	.	F	12
5	.	.	.	.	F	15
	.	.	.	.	F	13
	.	.	.	.	F	5
	.	.	.	.	M	3

* Data restricted to households having affected children aged 0–5 years.

Note. In practically every household other skin conditions were found in several members, for example, taenia versicolor, scabies, lice.

weeks after the initial examinations showed that most lesions had already disappeared. The history was that 'they had just dropped off'. It was difficult to obtain accurate histories from Fijian patients or their relatives but in the 1965 survey lesions were stated to have been present for periods from 1 to 12 months, with a mean of 4 months, and at the time of examination some were known to be resolving.

In all three surveys in Fiji there was evidence of spread of molluscum contagiosum within households. In 25% of households with the condition more than one member of the family was affected (Table 6).

DISCUSSION

The information from these two populations, distinct as to geographical location, climate, race and demography, complement one another in providing certain comparative factual data and a few speculative pointers relating to the natural history of molluscum contagiosum. In Scotland accurate history taking was limited by the selection of the population studied according to the condition under scrutiny. In Fiji, whilst histories were unreliable, entire populations were examined and the widely prevalent disease was studied in its natural setting.

The epidemiological pattern of molluscum contagiosum was different in these two groups. In Scotland peak incidence between 10 and 12 years contrasted with a peak at 2–3 years in Fiji. In Aberdeen males predominated, but the sexes were equally affected in Fiji. In Aberdeen the relationship between molluscum contagiosum and attendance of patients at public baths was striking, confirming the earlier findings of Walker (1910). The apparent anomaly that similar proportions of boys and girls swim, whilst more boys present with molluscum contagiosum, may be explained by the greater frequency with which boys indulge in this exercise. These findings fit well with the notion that the main factor which determines disease prevalence is opportunity for contagious exposure to the virus rather than any postulated inherent sex susceptibility. This opportunity occurs early in Fiji where the communal way of life, the relative lack of personal hygiene (see footnote, Table 6) and the scanty clothing, especially in the very young, contribute to early and repeated encounters with the virus. In the cooler, urban environment of Aberdeen, on the other hand, infection only becomes at all common in older children, under conditions which encourage, directly or indirectly, close bodily contact. Though chlorinated water in the swimming pool itself may well be viricidal, the opportunity for such infective contact during showering is great. Under normal conditions the virus must be of low intrinsic infectivity since spread within households and schools was very rare in Aberdeen, though 25 % of affected households in Fiji harboured more than one case. Also pointing to the importance of transmission by direct contact was the frequency with which lesions were encountered both unilaterally, suggesting auto-inoculation by scratching, and on opposing surfaces, as in the intergluteal cleft and on adjacent aspects of the axilla and upper arm. However, whilst these studies provide no direct evidence, the possibility is not excluded that within the human host molluscum virus may also spread during a phase of viraemia.

The central distribution of lesions in the Aberdeen survey was striking and was especially marked in the axilla. This is not in accord with the peripheral distribution generally described (Low, 1946; Ingram & Brain, 1957; Warren, 1965). However, in Fiji peripheral lesions were fairly common, being particularly prevalent on the lower limbs. Palms and soles were not affected except for one girl of 4 years, who had a single lesion at the junction of palm and wrist. Reasons for such discrepant distributions are not obvious, though the wearing of shoes and socks may be relevant. It is also possible that a critical skin temperature may be of importance in determining sites of virus growth.

In the absence of antibody studies the question of immunity in this condition must be speculative. However, three features seem worthy of note. First, no case was seen, either in Fiji or Aberdeen, under 1 year of age and only an occasional case presented over the age of 30. Although a prolonged incubation period may be of some importance, the possibility of antibody-mediated maternally-transmitted immunity should not be ignored. Secondly, after the first year of life, the age incidences in Fiji and Aberdeen are strongly reminiscent of the well-documented comparative age distributions of poliomyelitis infection, as determined both clinically and serologically, in Cairo and the United States before the days of mass immuno-prophylaxis against this condition (Paul, Melnick, Bennett & Goldblum, 1952; Paul, Melnick & Riordan, 1952). Thirdly, in Fiji, the suggestion of waning susceptibility with age, and the possibility that this may be immunologically mediated, receive tenuous support from the decreasing degree of skin involvement with age. The numbers of skin areas affected in the 1966 Fiji survey, in the age groups 0 to 5, 6 to 32 and greater than 32 years were, respectively, 4·8, 2·1 and nil. On the other hand, molluscum lesions undoubtedly recurred, either after natural resolution of earlier ones or after surgical excision of some. Since this latter procedure caused bleeding, the suggestion that trauma brings about natural resolution by releasing antigen to the blood with consequent antibody formation seems less likely.

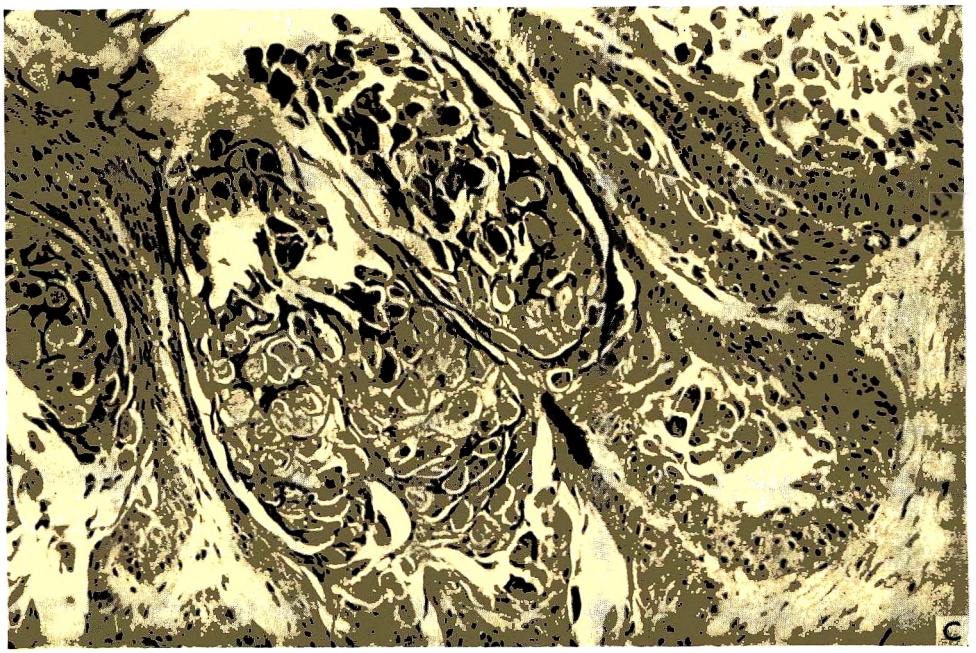
The difficulties encountered by practitioners in Aberdeen in identifying lesions of molluscum contagiosum may be a reflection of the relatively rare occurrence and benign nature of this condition. However those practitioners who do recognize it may well treat it themselves, and the incidence of molluscum contagiosum amongst hospital skin out-patients may not be a very reliable measure of its true incidence. In the years 1956–63 in Aberdeen, the incidence was 12/1000 skin out-patients. Low's figures in Edinburgh were 2/1000 in 1934–39 and 1·4/1000 in 1940–44. These findings contrast with the 4·5 % noted in an entire population of 672 persons in a village settlement in Fiji. Even this last figure represents only part of the truth since, as indicated, lesions disappear from the Fijian skin with remarkable rapidity. A more prolonged study is required, with repeated observations at short intervals, to plot the true natural history of this condition. Fiji would seem an ideal location to do this. Furthermore, an extension of these studies to include the racially and socially distinct Indian communities in Fiji would be of comparative interest.

SUMMARY

Between 1964 and 1966 comparative studies were carried out in Aberdeen, Scotland, and in village settlements in Fiji on the clinico-epidemiological features of molluscum contagiosum. In Aberdeen there was a positive correlation between this disease and attendance of patients at public swimming baths. The preponderance of male patients in Aberdeen was attributed to their more frequent indulgence in swimming. Household spread of the condition was rare in Aberdeen but common in Fiji. Lesions frequently occurred unilaterally or were situated on opposing skin surfaces. They were mainly central in distribution in Aberdeen,


0·25 μ
A


0·25 μ
B


C

the axilla being a site of predilection. In Fijians, peripheral lesions were fairly common though palms and soles were not affected. Peak age incidence in Aberdeen was 10–12 years, contrasting with a peak at 2–3 years in Fiji. Opportunity for contagious exposure appeared to be the main factor determining transmission of molluscum contagiosum between hosts, this opportunity occurring frequently and early in life in Fiji but only under special circumstances and later in childhood in Aberdeen. However, the age distributions in the two populations suggested the possible operation of immunological as well as environmental factors in determining the overall pattern of disease in the community.

We should like to express our thanks to the following people whose support and co-operation made this joint study possible: Dr K. J. Gilchrist, Principal of the Fiji School of Medicine; Prof J. A. R. Miles, Department of Microbiology, University of Otago; Dr C. H. Gurd, Director of Medical Services, Fiji; and Prof. A. Macdonald, Department of Bacteriology, University of Aberdeen. We are indebted to Dr T. E. Anderson and Dr R. A. Main of the Aberdeen Royal Infirmary for referring patients, to Mr W. Hodgkiss of the Torry Research Station, Aberdeen for carrying out the electron microscopy and to Dr Peter Bennett, Nuffield Foundation Scholar in Tropical Medicine from Aberdeen in 1962, who brought to the attention of the Aberdeen workers the prevalence of molluscum contagiosum in Fiji. Part of the work was supported by a grant to R. Postlethwaite from the British Empire Cancer Campaign for Research. Mr (now Dr) Ian Simpson and Miss Helen Adam were supported by Nuffield Foundation Scholarships in Tropical Medicine, and Dr J. A. Watt by a Garden Research Fellowship from the University of Aberdeen.

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EXPLANATION OF PLATE

A and B. Electron micrographs of extracts from molluscum lesions from patients in (A) Aberdeen and (B) Fiji.

C. Section of molluscum contagiosum lesion from Fijian patient stained by phloxine-tartrazine. × 150.

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Edwardsiella tarda in a study of juvenile diarrhoea

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Edwardsiella tarda is the name proposed by Ewing, McWhorter, Escobar & Lubin (1965) for a new group of organisms within the Enterobacteriaceae, studied since 1959 and referred to by them as 'bacterium 1483–59'. These bacteria were similar to those named the 'Asakusa' group by Sakazaki & Murata (1962) and Sakazaki (1965), and the 'Bartholomew' group by King & Adler (1964). The major characters which distinguish these organisms from existing genera are that they give a negative phenylpyruvic acid test and are mannitol-negative and produce abundant H_2S.

Of the human strains of Ewing *et al.* (1965) 25 out of 34 had been isolated from faeces, the others from extra-intestinal sites, but a history of diarrhoea was available in only five instances. One of the two animal strains in their study came from a bovine case of diarrhoea. The one strain described by King & Adler (1964) was isolated from the faeces of a patient with both enteric fever and acute gastro-enteritis. In contrast, of the 256 strains of Sakazaki (1965) 250 came from animals, almost wholly snakes, and only five from human gastro-enteritis cases. The incidence of *Ed. tarda* and its role in human diarrhoea is not precisely known, and it seems worth recording the isolation of such organisms during a special study of juvenile diarrhoea at present in progress at Vellore.

MATERIALS

Children below the age of 5 years in a rural area and an urban area were selected for a detailed bacteriological study during the period June 1963 to September 1965 to evaluate specifically the etiological role in juvenile diarrhoea of the Arizona, Citrobacter (including the Bethesda–Ballerup subgroup) and Providence genera of the Enterobacteriaceae. Also included were children with diarrhoea attending the paediatric outpatient service and those admitted to the children's ward of the Christian Medical College (C.M.C.) Hospital. Rectal swabs were taken from the diarrhoeal cases before antimicrobial therapy was begun.

A control group of children without diarrhoea in both the rural and urban areas were also investigated subsequently. Specimens collected monthly from non-

diarrhoeal children in the rural area were examined from September 1965 to January 1966 and in the urban area from September 1965 to July 1966.

METHODS

The isolation and identification methods used at C.M.C. were those of Bhat & Myers (1962) with a few modifications. The direct plating media were sheep blood agar (BA), MacConkey agar (MA), deoxycholate citrate agar (DA) and bismuth sulphite agar (BS). Enrichment in Selenite F broth was also used. After incubation overnight at 37° C., suspect colonies were subcultured to the set of preliminary screening tests shown in Table 1. These tests included the use of lysine iron agar (LIA) specifically recommended by Edwards & Fife (1961) for the identification of Arizona strains.

Table 1. *Reactions of* Edwardsiella tarda *in screening tests*

Test	*Ed. tarda* 4 strains
TSI: Butt (glucose)	AG
Slant (lactose/sucrose)	−
H_2S	+
LIA: Lysine decarboxylase	+
H_2S	+
Urease	−
Indole	+
Motility	+

AG = acid and gas.

The selected additional biochemical tests used at C.M.C. were similar to those included in the set of detailed tests (Carpenter, Lapage & Steel, 1966) in use at the Dysentery Reference Laboratory (D.R.L.), with minor exceptions. At C.M.C. nutrient broth with bromthymol blue as indicator was used for the fermentation tests, whereas at D.R.L. 1% (w/v) peptone water with Andrade indicator was used. Each medium contained 0·5% (w/v) of the various carbohydrates and in both laboratories the cultures were incubated for 21 days. The methyl red (MR) and Voges–Proskauer (VP) tests were incubated at 37° C. for 3 days at C.M.C., and for 3 days at 30° C. at D.R.L. For the phenylpyruvic acid (PPA) and malonate tests at C.M.C. the phenylalanine malonate medium of Difco Laboratories Inc., U.S.A., was used. In both laboratories the method of Møller (1955) was used for the decarboxylase tests.

RESULTS

During the period June 1963 to September 1965, from 1491 episodes of diarrhoea in 513 children (748 episodes in 265 rural children and 743 in 248 urban children) two proved strains of *Ed. tarda* and two presumptive strains (subsequently not available for detailed study) were isolated. No such strains were isolated from the control children without diarrhoea in the subsequent survey. The latter consisted

of 371 specimens from 141 rural children in the period September 1965 to January 1966, and 950 specimens from 178 urban children from September 1965 to July 1966. (Details of other intestinal pathogens will be published elsewhere.)

Clinical history of patients

Case 1. This was an 8-month-old male from the urban area with a history of having watery greenish yellow stools without blood and mucus for 3 weeks before the organism, subsequently identified as *Ed. tarda*, was isolated from a faecal specimen in December 1963. Other recognized intestinal pathogens were not isolated on this occasion. Apart from this episode of diarrhoea, the child had had eight others during the period of study, one of which had been associated with the known pathogen, *Salmonella enteritidis*.

Case 2. This was a 16-month-old female child from the rural area situated about 14 miles from the urban area in the study. This child, when first examined, had a history of diarrhoea of 3 days' duration, and a rectal swab yielded *Shigella flexneri* 2*a*. From the follow-up specimen, taken 2 weeks later in December 1963 when the child had recovered from her diarrhoea, the shigella strain was not isolated again, but the strain of *Ed. tarda* was cultured, and so this strain was isolated during convalescence from bacillary dysentery. During the period of the study, this child had had six episodes of diarrhoea, two of which had been associated with shigella infections, but specific pathogens had not been isolated during the other four attacks

Cases 3 *and* 4. These were a 4-year-old male child and a 3-year-old female child in two nearby hamlets in the rural area under study, both children having a history of diarrhoea for 3 days. The presumptive strains of *Ed. tarda* were unlikely to be directly linked epidemiologically as they were isolated from the children in October 1964 and January 1965 respectively. *Sh. flexneri* 2*a* was isolated from the female child at the same time.

Reactions of the strains

The initial biochemical and serological examination of the first two strains, isolated in 1963, suggested that the strains did not belong to any of the recognized genera of the Enterobacteriaceae, and the report of a reference laboratory to which they were submitted confirmed this, but the organisms were not specifically identified. However, in 1966, a review of the reactions of the strains by one of us (K.P.C.*), in consultations at Vellore, strongly indicated that the strains might be *Ed. tarda*, and subsequently the strains were examined in detail to confirm their identification. At this time, unfortunately, only the strains from the first two cases were viable.

Colonial appearance

On BA the colonies of the four strains were small, greyish, smooth, low dome-shaped and non-haemolytic. On MA and DA they were small and pale non-lactose-fermenting and after incubation for 48 hr. the colonies on DC developed a dark

* During the tenure of a W.H.O. Exchange of Research Workers Grant.

centre. On BS they were greyish with a tiny brownish halo and metallic sheen. The colonies were rather smaller than salmonellas or shigellas on comparable media. All strains survived enrichment in selenite broth.

Biochemical reactions

The reactions of the four strains in the screening tests are given in Table 1. Table 2 combines the detailed reactions of the first two strains and some of the reactions of the second two strains in the tests done at C.M.C. and D.R.L.

Table 2. *Detailed biochemical reactions of* Edwardsiella tarda

Test	*Ed. tarda*	Test		*Ed. tarda*
Glucose	AG	Simmons's citrate		–
Lactose	–	Christensen's citrate		+
Mannitol	–	Malonate		–
Maltose	AG	Gluconate		–
Glycerol	(*a*)	Gelatin liquefaction		–
Sucrose, dulcitol, salicin, xylose, adonitol, arabinose, cellobiose, dextrin, inositol, raffinose, rhamnose, sorbitol, trehalose	–	Growth in KCN		–
		Nitrate reduction		+
		β-galactosidase		–
		Catalase		+
		Oxidase		–
PPA	–	Decarboxylases:	Arginine	–
MR	+		Lysine	+
VP	–		Ornithine	+
		Hugh & Leifson		Fermentation

(*a*) = weak acid production after incubation for more than 48 hr.

Serological reactions

As the reactions in the screening medium, LIA, were similar to those of the *Salmonella* and Arizona groups, the strains were specifically tested in polyvalent salmonella O sera covering groups A to E (Lederle Laboratories, U.S.A.) and in seven pools of Arizona O sera (kindly provided by Dr Joan Taylor, Colindale) but the four strains did not agglutinate in these sera nor in the sera available for any other recognized pathogenic group of the Enterobacteriaceae. Subsequently, Dr R. Sakazaki, Tokyo, typed the first strain (F197/63) as *Ed. tarda* 016: H1, and the second strain (F236/63) as H4 but the O group could not be determined as the strain was by then serologically rough.

Drug sensitivity pattern

The strains were sensitive to chloramphenicol, streptomycin, tetracycline, neomycin, ampicillin and furazolidone but resistant to sulphonamides and colomycin.

DISCUSSION

Strains of *Ed. tarda* were isolated from only four out of 832 children under five years during an intensive survey extending over approximately 3 years. These strains were isolated from children with diarrhoea or an immediately antecedent history of it, and none was isolated from the control children without diarrhoea.

From one of the children whose specimen yielded this organism the accepted pathogen, *Sh. flexneri* 2*a*, was isolated in addition, and another child was convalescent from a shigella infection. Dorigan & Deinhardt (personal communication) likewise reported the simultaneous isolation of *Sh. sonnei* and *Ed. tarda* from a marmoset. It is not possible from the C.M.C. study to decide whether *Ed. tarda* is a specific pathogen in its own right or a secondary invader in an otherwise physiologically abnormal intestine. However, it would appear from this study that this organism is rare in human intestinal infections and indeed no other wild strains from patients in either Britain or elsewhere have been received by D.R.L. from 1945 to 1966. It is possible from the published records of the isolation of this organism (King & Adler, 1964; Ewing *et al.* 1965; Sakazaki, 1965; Wallace, White & Gore, 1966) that it may have a specific geographic distribution possibly dependent on the reservoir being mainly in such animals as snakes, seals and alligators, and that man is only an accidental host. This is analogous to the Arizona group as no human infections caused by these organisms have been recorded in Britain up to the end of 1965 (J. Taylor, personal communication).

The reactions given in Tables 1 and 2 conform to those of stock strains of *Ed. tarda* (kindly supplied by Dr W. H. Ewing, Atlanta, U.S.A.) and to those of other published reports.

From Table 1 it is clear that the reactions of *Ed. tarda* in the screening tests are similar to those of the *Salmonella* and Arizona groups except for the indole reaction, though indole-positive salmonella strains do occur. These groups typically show rapid decarboxylation of lysine and abundant H_2S production in LIA, and in this study the LIA medium proved a very useful screening medium for the various H_2S-producing organisms of the Enterobacteriaceae which give identical reactions in triple sugar iron (TSI) agar.

Ed. tarda ferments very few carbohydrates but in its other biochemical reactions it gives, apart from H_2S production, the basic pattern of *Escherichia coli*, or the Alkalescens-dispar group which it closely resembles by its reactions in the two citrate media. As it ferments so few carbohydrates, and particularly fails to ferment mannitol, it may be superficially confused with strains of *Proteus* and Providence from which it is easily distinguished by its negative urease and PPA reactions, and with *Plesiomonas shigelloides* (Eddy & Carpenter, 1964) from which it is distinguished by the negative oxidase reaction.

Though *Ed. tarda* appears to be rare in human intestinal infections it is possible that it is not being recognized as it has been only recently described and has unusual characters.

The accepted major pathogens of the Enterobacteriaceae are isolated from only a small proportion of patients with diarrhoea, and it is urged, therefore, that more specific attention should be paid to the isolation of other enterobacteria, such as *Ed. tarda* for example, to determine further their etiological role in intestinal infections.

SUMMARY

Four strains of *Edwardsiella tarda*, a recently described member of the Enterobacteriaceae, were studied, They were isolated from four out of 832 children during a 3-year survey of juvenile diarrhoea at Vellore, S. India. Three of the four children had diarrhoea when the organisms were isolated and one was convalescent from bacillary dysentery. From this study and a review of the literature it appears that *Ed. tarda* is found only infrequently in man and that its main reservoir may be in animals such as snakes and seals. It is suggested that this organism should be specifically looked for in cases of diarrhoea to determine its etiological role, particularly when the major intestinal pathogens are not isolated.

This work forms part of the studies on the etiology of diarrhoeal infections in children for the Ph.D thesis of P.B., and the grant from the Indian Council for Medical Research towards these studies is gratefully acknowledged. We also wish to thank Dr Sakazaki for serotyping the strains.

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Mercury resistance and tetracycline resistance in *Staphylococcus aureus*

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INTRODUCTION

Since resistance to mercury salts was first described as a useful marker for identification of epidemic strains of *Staphylococcus aureus* (Moore, 1960), attempts have been made to correlate mercury resistance with other physiological markers. Relationships have been described between mercury resistance, tetracycline resistance and multiple antibiotic resistance (Turner & Willis, 1962; Willis & Turner, 1963; Willis, Jacobs, & Goodburn, 1964), and between mercury resistance, high penicillinase production, tetracycline resistance and multiple antibiotic resistance (Richmond, Parker, Jevons & John, 1964).

Most workers have noted that mercury resistant strains are found predominantly among staphylococcal strains of phage groups I and III, and particularly among those of the 'pure 80/81 strains' (Parker & Jevons, 1963), and among strains of type 83A (Green, 1962; Jessen *et al.* 1963). Parker & Jevons (1963) also noted the close relationship between mercury and tetracycline resistance, and the multiple antibiotic-resistant nature of mercury resistant strains has been confirmed by Vogelsang (1965).

This paper is concerned with the relationship between mercury and tetracycline resistance. Mercury resistant staphylococci are usually, though not invariably, resistant to tetracycline, whereas tetracycline resistance occurs only sporadically in mercury sensitive strains. Evidence is produced which shows a qualitative difference between the tetracycline resistance of the two groups. Tetracycline resistance in mercury resistant strains is regarded as genetic in origin—the result of mutation, followed by selection. Resistance to tetracycline in the mercury sensitive strains is possibly the result of 'training' or adaptation to the drug.

MATERIALS AND METHODS

Staphylococcal strains

Tetracycline resistant strains were obtained from a number of different sources. All strains were independently isolated and differed in phage pattern, antibiogram or place of isolation. The 119 strains in survey I consisted of 101 staphylococci isolated at St Thomas's Hospital over a period of about $2\frac{1}{2}$ years in the course of other investigations, and 18 strains (supplied by the Central Public Health Laboratory, Colindale) which had caused outbreaks of sepsis in hospitals before 1963.

The 137 strains in survey II were also supplied by the Central Public Health

Laboratory and were selected from the annual surveys of 1965 and 1966. These surveys are carried out on staphylococci isolated consecutively over a given period in a number of hospitals in the London area.

All strains but one were resistant to both penicillin and tetracycline. Penicillin sensitive strains were excluded because of the close genetic linkage between mercury resistance and penicillinase production (Richmond & John, 1964) as it was not possible to decide whether these strains had originally been resistant to mercury or not. The one penicillin sensitive exception had the phage pattern 3C/55/71: phage group II strains are generally mercury sensitive (Green, 1962) and, as only three other tetracycline resistant examples from this group were found, this strain was retained.

Mercury sensitivity and antibiograms

Mercury sensitivity was tested by the method of Green (1962). Antibiotic sensitivity was tested by applying 'Multodisks' (Oxoid) to surface lawns of growth on nutrient agar ('Oxoid' Blood agar base, no. 2). The composition of the multodisks was: tetracycline, 50 μg.; chloramphenicol, 50 μg.; erythromycin, 50 μg.; neomycin, 30 μg.; novobiocin, 30 μg.; cloxacillin, 5 μg.; streptomycin, 25 μg.; penicillin, 5 units.

Phage typing

Phage typing was carried out by the method of Blair & Williams (1961).

Resistance of strains to tetracycline

Minimum inhibitory concentration (M.I.C.) of tetracycline for each strain was estimated by absence or presence of growth on nutrient agar containing the antibiotic. Doubling dilutions of pure tetracycline hydrochloride (Lederle) were incorporated in 20 ml. amounts of nutrient agar to give a range of concentrations of 3·125 μg./ml. to 200 μg./ml. All plates were freshly prepared. After drying, these were inoculated by application of overnight broth cultures with a multiple loop device, and incubated at 37° C.

Growth curves

Growth patterns of selected strains in a therapeutic concentration of tetracycline (3 μg./ml.) (Barber & Garrod, 1963) were investigated turbidimetrically in an EEL nephelometer (Evans Electroselenium Ltd.). Strains were selected to provide two or more representatives of each of a series of M.I.C.'s tetracycline in the mercury resistant and mercury sensitive groups (Table 1).

Metal-capped 6 in. $\times$ $\frac{1}{2}$ in. test tubes containing 10 ml. peptone water (Evans peptone, 1 %; sodium chloride, 0·5 %; w/v) and tetracycline hydrochloride, 3 μg./ml., were each inoculated with 0·02 ml. of an overnight peptone water culture (approximately 50,000 viable organisms). Control tubes containing peptone water only were inoculated at the same time. Peptone water was selected as a culture medium so that growth would not occur too rapidly.

Tubes were incubated at 37° C. and turbidity readings taken hourly from 4 to 12 hr., and again at 24 hr. Because of the relative insensitivity of the nephelo-

meter during the early period of growth, readings were not consistently recordable until 4 hr. incubation had elapsed.

Table 1. *Strains selected to represent different M.I.C.'s of tetracycline in growth curve measurements*

M.I.C. tetracycline (μg./ml.)	Mercury resistant strains. Phage pattern	Mercury resistant strains. Phage pattern
100	52/52A/80/81	52/52A/80/81
100	83A	77
100	47/53/75/77	—
50	80*	29/52/52A/79/80
50	B5	71
50	80/47*	77*
50	—	3C
25	80*	29/80/47/53/75
25	53*	42D/81
12·5	—	N.T.†
12·5	—	3C/55/71‡
Totals	8 strains	10 strains

* Reactions with phages at 1000 × R.T.D. (1000 × Routine Test Dilution of phage).
† This strain died during training to growth at high levels of tetracycline.
‡ Penicillin sensitive strain.

Readings were recorded as logarithms to base 2 according to the tables of Finney, Hazlewood & Smith (1955), and the turbidities were checked against viable counts (Miles & Misra, 1938) as recommended by Monod (1949).

Two sets of growth measurements were taken on the selected strains. The first set was obtained from staphylococci that had not been pretreated in any way. The second set was made from the same strains after they had been adapted to grow on agar containing high levels of tetracycline (100 μg./ml.).

Adaptation of strains

Adaptation was initiated by plating the staphylococci on agar containing tetracycline, 3 μg./ml., and thereafter on increasing concentrations until all the strains grew on agar containing tetracycline, 100 μg./ml. On completion of this process, the strains were grown in plain peptone water overnight, and the growth pattern investigated as described on the following day.

One nontypable strain, M.I.C. tetracycline 12·5 μg., died during adaptation to growth at higher concentrations of the antibiotic.

RESULTS

The staphylococci in both surveys were divided into two groups—mercury resistant and mercury sensitive.

Distribution of minimum inhibitory concentrations of tetracycline

Percentage distribution of different levels of M.I.C. tetracycline in each group in both surveys are shown in Fig. 1. The mean percentage distributions of M.I.C.'s for all strains studied are shown in Fig. 2.

Resistance to tetracycline in both surveys is generally higher in the mercury resistant than in the mercury sensitive strains. The two sets of mercury resistant strains show a close similarity in distribution of resistance to tetracycline, and the lower distribution of M.I.C.'s in the mercury sensitive strains shows close corres-

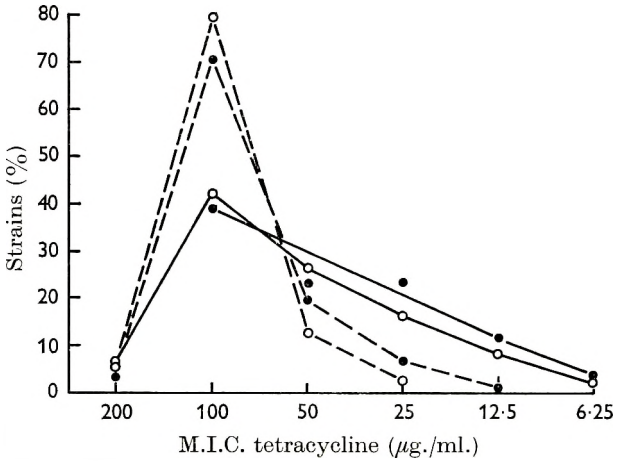

Fig. 1. Distribution of M.I.C.'s tetracycline in strains of survey 1 and survey 2. Survey 1: ● - - - ●, mercury resistant strains (93); ●——●, mercury-sensitive strains (26). Survey 2: ○ - - - ○, mercury resistant strains (87); ○——○, mercury sensitive strains (50).

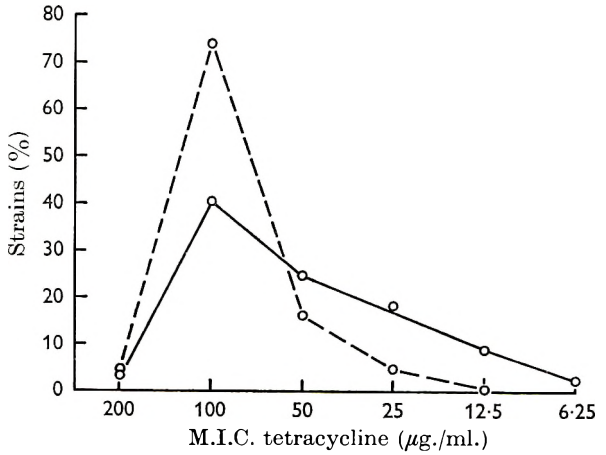

Fig. 2. Mean distribution of M.I.C.'s tetracycline for all strains (surveys 1 and 2). ○- - -○, mercury resistant strains (180); ○——○, mercury sensitive strains (76).

pondence in the two surveys. The number of mercury sensitive strains in survey 1 is considerably smaller than in survey 2. Nonetheless, there is no significant difference between these groups in both surveys.

Differences in distribution of M.I.C.'s tetracycline between mercury resistant and mercury sensitive strains are clearly seen if an arbitrary level (tetracycline, 50 μg./ml.) is taken to divide all the strains studied into those of high resistance and those of lower resistance (Table 2). The mercury resistant group has a pre-

ponderance of strains (70–85 %) resistant to tetracycline, 100 or 200 μg./ml. The majority of mercury sensitive strains (50–62 %) have an M.I.C. tetracycline of 50 μg./ml. or less.

Table 2. *Percentage distributions of M.I.C.'s tetracycline in mercury resistant and mercury sensitive strains*

	Mercury resistant			Mercury sensitive		
M.I.C. tetracycline	Survey 1	Survey 2	Mean	Survey 1	Survey 2	Mean
100 and 200 μg./ml.	73·2	84·9	78·8	38·5	48	44·7
50 and < 50 μg./ml.	26·7	15	21·1	61·5	52	55·2
Totals	99·9	99·9	99·9	100	100	99·9

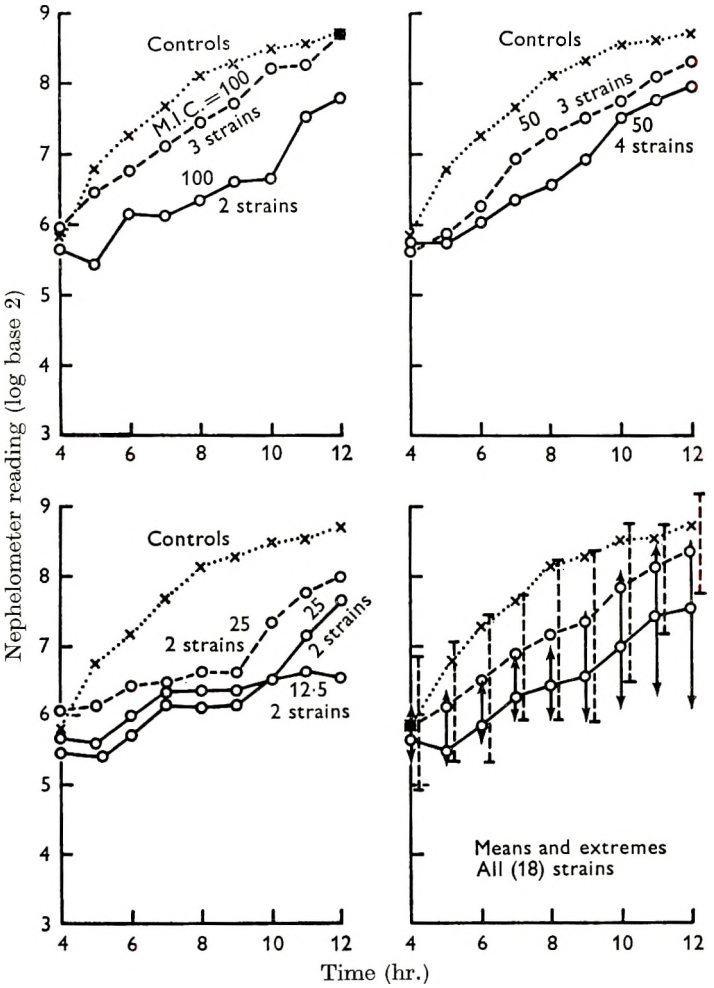

Fig. 3. Growth of representative strains (see Table 1) in peptone water containing tetracycline, 3 μg./ml. These strains have not been 'trained'. × . . . . ×, Controls; ○——○, mercury sensitive strains; ○ - - - - ○, mercury resistant strains.

Growth curves in therapeutic concentrations of tetracycline

Results of the growth curves of untrained strains in a therapeutic concentration of tetracycline (3 μg./ml.) are shown in Fig. 3. The curves cover the period of growth from 4 to 12 hr. Mean growth rates for the strains representing the various M.I.C.'s tetracycline—see Table 1—are plotted so that the mercury sensitive and mercury resistant groups can be compared. Mean growth rates for all the strains, together with extremes of readings are also shown.

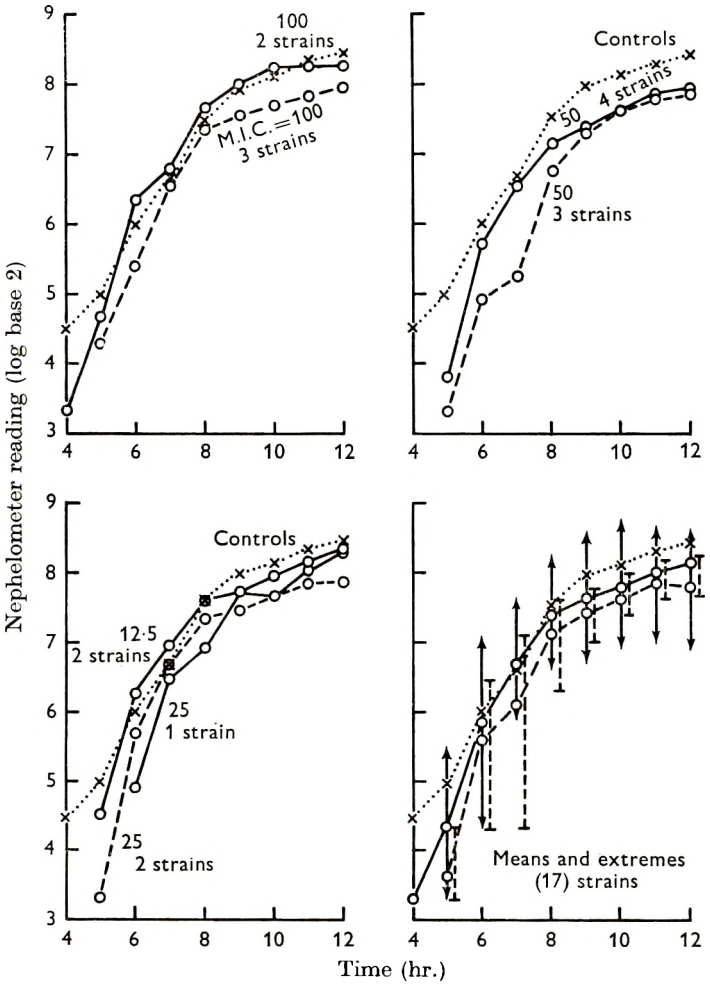

Fig. 4. Growth of representative strains in peptone water containing tetracycline, 3 μg./ml., after they had been 'trained' to grow at high concentrations of tetracycline. × ..... ×, Controls; O——O, mercury sensitive strains; O— —O, mercury resistant strains.

Controls are plotted as means of all readings taken during growth of the strains in plain peptone water: in terms of viable counts, control readings reached a maximum of about $2{\cdot}5 \times 10^6$ organisms/ml. in the 12 hr. period of incubation.

Mercury resistant strains show a gradual decline in growth rate with decrease in

resistance to tetracycline, but, even so, each group of mercury resistant strains grows better than the corresponding mercury sensitive groups representing each M.I.C. tetracycline.

Apart from the difference in growth rate, mercury sensitive strains show an appreciable delay in reaching their maximum growth rate in comparison with the mercury resistant strains. Such a delay is often found during 'training' or adaptation of bacteria to drug resistance (Dean, personal communication). The possibility of adaptation as a mechanism of tetracycline resistance in these mercury sensitive strains is supported by the incubation period, which is not sufficiently long to permit any appreciable selection of tetracycline resistant mutants that may have arisen spontaneously (Dean & Hinshelwood, 1966).

The readings for growth of untrained strains up to 12 hr. were analysed by the 'T' test (Industrial Experimentation, War Office, H.M.S.O., 1949 reprinted 1960, page 35). The T value is 2·33 (S.D. = 5·4). This T value is for 16 degrees of freedom significant at the 5% level of confidence ($P < 0{\cdot}05$).

Readings taken at 24 hr. show a wide scatter in both groups, and there is no consistent difference between mercury resistant and mercury sensitive strains.

The growth curves for the same strains after adaptation to growth on agar containing tetracycline, 100 μg./ml. (with the one exception that died during 'training'—see Table 1) are shown in Fig. 4. After 'training' the growth rate of the mercury sensitive strains is noticeably increased in the therapeutic concentration of tetracycline, and they now grow somewhat better than the mercury resistant strains.

Furthermore, there is no longer any obvious difference between the growth patterns of the controls—which are the same 'trained' strains grown in plain peptone water—and the two groups under investigation. The marked alteration in the growth pattern of the mercury sensitive strains suggests a more complete adaptation to growth in the presence of tetracycline.

Mercury resistant strains also show some difference in growth pattern after 'training', though these are not so pronounced as in the mercury sensitive group. The slope of the curve is now steeper, suggesting that growth has commenced earlier than in the untrained strains, and the extremes of readings during the growth of the various strains are now closer together. However, allowing for experimental variation, there is no great alteration in the pattern of growth of mercury resistant strains following 'training'.

DISCUSSION

A number of associations of mercury resistance in *Staphylococcus aureus* have been described by different workers. These include tetracycline resistance, high penicillinase activity, multiple resistance to antibiotics and susceptibility to lysis by phages of groups I and III. A further relationship, albeit incomplete, has been suggested between mercury resistance and ability to withstand drying (Rountree, 1963).

These factors are of undoubted importance in ensuring survival of strains within

the hospital environment, and possession of all or most of these markers ensures the success of 'hospital' or epidemic staphylococci. Two markers that are virtually constant in these strains are mercury resistance and tetracycline resistance.

The clinical behaviour of mercury resistant strains (Moore, 1960) has been further studied by Jessen *et al.* (1963), who reported that a higher mortality rate (50 %) results when the strains causing bacteraemia are mercury resistant than when the infecting strains are mercury sensitive (33 %). Richmond *et al.* (1964) showed that 31 out of 38 strains responsible for endemic sepsis were mercury resistant and, of these, 29 were tetracycline resistant.

The importance of tetracycline resistance as a marker of hospital strains has long been recognized. Many reports (Rountree & Thomson, 1952; Clarke, Dalgleish & Gillespie, 1952; Lowbury, Topley & Hood, 1952; Shooter *et al.* 1958; Williams, 1959; and Barber *et al.* 1960) have emphazised the dangerous nature of tetracycline resistant strains, their emergence and predominance in cases of hospital sepsis, and their ability to spread by cross-infection.

Tetracycline resistance alone, however, is not a reliable marker for recognition of hospital strains, as many non-epidemic strains now possess this characteristic, though there may be a qualitative difference between tetracycline resistance in mercury resistant and mercury sensitive strains, as the results given in this paper suggest.

Tetracycline resistance in mercury sensitive strains is shown to be generally lower than in the mercury resistant group. This finding suggests that strains sensitive to mercury have a less efficient mechanism for developing resistance to tetracycline under natural conditions, and the growth patterns suggest that the appearance of resistance is the result of 'training' or adaptation to the drug.

This argument is supported by the different behaviour of the mercury sensitive strains after they have been 'trained' to grow at high concentrations of tetracycline. Exponential growth occurs much sooner than in the same strains before 'training', and the growth rate increases.

The mechanism of 'training' of bacteria to drug resistance has been studied by Dean & Giordan (1964) who trained *Bact. lactis aerogenes* at various concentrations of terramycin. They demonstrated the continuous gradation of the resistance of 'trained' strains to the training concentration of the drug, and pointed out that the lag in growth in the presence of the antibiotic was shortened with each stage of training. They considered that 'training' was complete when no further appreciable shortening of the lag occurred in a given concentration of antibiotic.

In the present work, the concentration of tetracycline used in 'training' is far in excess of any that the bacteria would meet under actual hospital conditions, and it is apparent that, once the ability to become resistant has been developed—whatever the mechanism of that resistance—the degree of resistance can be greatly increased.

The changes in behaviour of the mercury resistant strains as a result of 'training', however, are not so marked as in the mercury sensitive staphylococci that have undergone the same treatment. The training process has less effect on the growth rate of the mercury resistant strains, and this suggests that their resistance is less

dependent on the presence of antibiotic in the environment, and is probably genetically determined.

The effect of 'training' on the mercury sensitive strains does not, however, preclude the possibility of mutation being the starting point in development of resistance to tetracycline. Drabble & Hinshelwood (1961), in investigations of streptomycin resistance in *Bact. lactis aerogenes*, described preliminary selection of pre-existing mutants at low concentrations of the drug. These mutants could then be 'trained' to become more resistant, and the degree of resistance finally achieved was dependent on the concentration of streptomycin used in 'training'.

A similar process could account for tetracycline resistance in mercury sensitive strains of staphylococci isolated from clinical sources. The resistance in these strains before 'training' is stable, as shown by repeated estimation of M.I.C. tetracycline, but this is not surprising, as the relative stability of adaptive changes in bacteria has long been recognised (Penfold, 1910). The degree of resistance to tetracycline of the mercury sensitive strains may well be a reflection of the amount of the drug used in the environment from which they were isolated.

Because of the clinical importance of epidemic staphylococci, genetic studies have been largely confined to strains having their characteristics, and little attention has been directed to the mechanism of resistance in the other groups.

Studies of genetic markers in resistant staphylococci have shown that the genes responsible for mercury resistance and penicillinase production are closely associated (Richmond & John, 1964) and can be co-transduced to sensitive strains; and that the genes responsible for tetracycline resistance and penicillin resistance are probably carried cytoplasmically and are found on different plasmids (May, Houghton & Perret, 1964) and particularly among strains of the '52, 52A, 80, 81' complex (Asheshov, 1966). However, these reports refer to strains that are undoubtedly 'hospital' staphylococci.

Published reports of transduction of tetracycline resistance suggest that successful transduction has been achieved from epidemic (or mercury resistant) donor strains. Only one author (McDonald, 1966) states that the donor strains were mercury resistant. However, descriptions of donor organisms in other studies (Mitsuhashi, Nakano, Fukutome & Kakinuma, 1961; Pattee & Baldwin, 1961; Collins & McDonald, 1962; and Mitsuhashi, Oshima, Kawaharada & Hashimoto, 1965) though incomplete in many respects, suggest that tetracycline resistance was transduced from mercury resistant donor strains or, at least, from strains that had the characteristics of epidemic staphylococci. No reports have been found in which transduction is described as being carried out from strains known to be mercury sensitive.

Further study on the nature of tetracycline resistance in mercury sensitive strains is required. A comparison between the incidence of tetracycline resistance in these strains and the amount of tetracycline used in the environment would be of interest.

The mercury test (Green, 1962) is easily performed and yields valuable information. Its use as a routine test does not demand additional media or glassware as it can be performed on the same plate as a 'Multodisk' test involving eight anti-

biotics. The information so gained is essential in genetic and epidemiological studies, and descriptions of staphylococcal strains in published reports should include mercury resistance or sensitivity.

SUMMARY

Minimum inhibitory concentrations of tetracycline to 256 tetracycline-resistant strains of *Staphylococcus aureus* were determined. M.I.C.'s tetracycline were appreciably higher among mercury resistant than among mercury sensitive strains.

Mercury resistant strains representing various M.I.C.'s tetracycline grew significantly better in peptone water containing a therapeutic concentration of tetracycline than mercury sensitive strains representing the same range of resistance. The experiment was repeated after both groups had been adapted—or 'trained'—to grow on agar containing tetracycline, 100 μg./ml. The mercury sensitive strains now grew better than the mercury resistant group.

The significance of these findings is discussed. It is concluded that tetracycline resistance is more stable and efficient in mercury resistant strains, and that it is probably genetic in origin—the result of mutation and selection. Tetracycline resistance in mercury sensitive strains is possibly the result of 'training'.

The associations and significance of both mercury resistance and tetracycline resistance in *Staphylococcus aureus* are discussed.

The author is indebted to Dr M. T. Parker, for supplying many of the strains studied; to Dr A. C. R. Dean for help in interpreting the growth curves; to Dr Dennis Pym, Birkbeck College, W. C. 1 for statistical analyses; to Miss Jennifer Bambury for technical assistance and to Lederle Laboratories for a gift of tetracycline hydrochloride.

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Phage typing of *Staphylococcus aureus* from dairy cattle in Australia

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INTRODUCTION

Phage typing is an established technique in epidemiological studies of *Staphylococcus aureus* infection in man. The technique has also been used on animal strains, particularly those from dairy cattle, in which mastitis due to this organism is a serious problem in all the major dairying countries.

Some workers have used the 'human' phages to type bovine strains (Macdonald, 1946; Smith 1948*a*; Price, Neave, Rippon & Williams, 1954); others have isolated new phages where the human phages were unsatisfactory (Smith, 1948*b*; Seto & Wilson, 1958; Coles & Eisenstark, 1959*a*, *b*; Nakagawa, 1960). Davidson (1961) carried out an intensive study, and a set of phages suitable for typing bovine strains was suggested.

This paper records observations of phage typing of bovine strains in Australia, with the subsequent selection of a series of phages for typing such strains.

MATERIALS AND METHODS

Staph. aureus was isolated from milk samples collected mainly from herds in the Brisbane milk supply area. All strains were coagulase positive when tested by the tube method with rabbit plasma diluted 1/10.

The basic technique used in phage typing was as described by Blair & Williams (1961). The human set of phages (termed 'human phages') was obtained from Dr P. Rountree, Royal Prince Alfred Hospital, Sydney. New phages were isolated by the cross-culture method of Fisk (1942). Nine additional phages were obtained from the Central Veterinary Laboratory, Weybridge, U.K., propagated and tested (Davidson, 1961). Phages were used at routine test dilution (RTD) for typing; a machine similar to that described by Tarr (1958) was used.

Preliminary typing using the human phages (29, 52, 52A, 79, 80, 3A, 3B, 3C, 55, 71, 6, 7, 42E, 47, 53, 54, 73, 75, 77, 31B, 47D, 42D, 81, 187) was carried out by Miss Y. Battey of the Department of Health, Brisbane. This was the recommended human set (Blair & Williams, 1961) with the addition of 73, 31B, and 47D, three group III phages. These phages were all used at RTD.

RESULTS

Results of typing with human phages

The effect of the human phages was assessed from the typing of 1820 strains of *Staph. aureus*, isolated from 51 herds. Forty-four of these herds were sampled once for a survey (Frost, 1962). The remaining strains came from seven herds in which control of mastitis due to *Streptococcus agalactiae* was studied; these herds were sampled frequently and many strains of *Staph. aureus* were isolated and

Table 1. *Phage typing of bovine strains of* Staph. aureus *with the human phage set*

	Phage group						
Origin	I	II	III	IV	Misc.	Non-typable	Total
44 survey herds*	144	8	338	9	21	329	849
7 control herds*	9	—	94	57	18	102	280
Total	153	8	432	66	39	431	1129
Percentage	13·6	0·7	38·3	5·8	3·5	38·2	—
All strains tested							
Total	172	10	603	308	155	572	1820
Percentage	9·5	0·5	33·1	16·9	8·6	31·4	—

* The typing of strains from a single herd sampling only are considered. Phages used at RTD.

Table 2. *The classification of phage patterns from* 19 *survey herds in which* 20 *or more strains were examined*

	Phage group						
Herd no.	I	II	III	IV	Misc.	Non-typable	Total
1	3	—	18	1	—	8	30
5	4	—	19	—	—	11	34
6	6	—	1	4	—	9	20
8	7	—	3	—	—	22	32
10	24	—	37	—	—	2	63
11	2	1	17	—	—	11	31
12	—	—	6	—	—	23	29
13	8	—	—	—	—	29	37
17	1	2	7	—	2	9	21
18	18	—	2	—	—	8	28
21	6	—	18	—	—	4	28
23	—	—	3	—	—	26	29
25	4	—	17	—	1	24	46
28	2	—	12	—	1	5	20
34	9	—	15	—	—	21	45
35	—	—	9	2	1	18	30
36	—	—	17	—	2	7	26
38	—	—	31	—	—	4	35
42	1	—	8	—	—	18	27
Total	95	3	240	7	7	259	611
Percentage	15·5	0·5	39·3	1·1	1·1	42·4	—

Phages used at RTD.

typed. Results are summarized in Table 1. For this classification, staphylococci were allotted to phage groups as defined by Parker (1962).

However, unless the proportion of strains typed is assessed in relation to the herd, these results can be misleading. Table 2 shows the classification of strains typed on a herd basis, where 42·4 % of staphylococci were non-typable. The proportion of non-typable strains was too high in some herds to differentiate the strains present.

Table 3. *The lysis of* 336 *staphylococci, non-typable with the human phage set at RTD, with* 19 *additional phages*

Phage and origin		No. of strains lysed	Percentage
78		1	0·3
31B		1	0·3
101	Davidson (1961)	5	1·4
102		227	82·4
107		57	17·0
108		311	92·6
110		5	1·4
111		317	94·3
115		0	0·0
10	Brisbane	299	68·2
11		3	0·9
12		2	0·6
13		3	0·9
186		268	80·0
221		241	71·7
367		254	75·6
373		234	69·6
425		291	86·6
600		239	71·1

Phages used at RTD.

Table 4. *The number of phages by which each strain was lysed*

(From the typing of 336 strains shown in Table 3.)

No. of phages causing lysis...	11	10	9	8	7	6	5	4	3	2	1	Total
No. of strains	29	97	68	28	15	31	33	13	3	10	9	336
Percentage	8·6	28·9	20·2	8·3	4·5	9·2	9·8	3·9	0·9	3·0	2·7	100

The use of other phages

A number of new phages were isolated, propagated, and used at RTD to type 'non-typable' strains. Preliminary results with eight new phages showed that only 13 of 100 selected strains, non-typable with the human phages above, were not lysed. Then 376 such non-typable strains were typed at RTD with 19 phages; these consisted of ten new phages and nine from the bovine set of Davidson (1961). In general, these phages were either very active, lysing many of these staphylococci, or else lysed only a small number. Forty staphylococci were still not lysed by any phage, but since these were derived from a group of 1820 strains originally

examined, it was clear that a combination of the human phages and selected bovine phages would lyse a high proportion of bovine staphylococci. The results on the 336 strains which were lysed are shown in Tables 3 and 4.

In order to compare the phages under similar conditions, a series of 1404 strains of staphylococci was then typed with 42 phages, using two lawns of each strain and two sets of phages, as follows:

Human phages	Bovine phages
29, 52, 52A, 79, 80	78, 31B, 101, 102, 107
3A, 3B, 3C, 55, 71	108, 110, 111, 115, 221
6, 7, 42E, 47, 53,	373, 10, 11, 12, 13,
54, 75, 77, 42D, 81	186, 1050, 1054, 367, 425
187	600

The first nine of the bovine set of phages were those recommended by Davidson (1961). The remainder comprise the ten Brisbane phages shown in Table 3, together with two other phages of local origin (nos. 1050 and 1054).

There were 94 (6·7 %) strains which were non-typable with all 42 phages, 417 (29·7 %) were non-typable with the human phages but were lysed by one or more of the bovine phages. Twenty-seven strains which were typed by the human phages were non-typable with the bovine phages. Of these strains, nine were lysed by phage 53, one by phage 80 and seven were lysed by phages 80 and 81. The remainder were lysed by phages of group III (seven different patterns) and group II (three different patterns). Phage 187 did not lyse any of the 3224 staphylococci which were typed in these and the previous observations.

As before, many of these strains were from a limited number of control herds, and the value of the phages was assessed mainly from the typing of 397 of these strains from 328 herds; 280 of these strains were isolated from bulk milk. Not more than two strains were included from any one herd and these always belonged to different phage groups. Eighty-one strains were not lysed by the human phages, but were lysed by one or more of the bovine phages. A further 32 strains lysed by one or more bovine phages gave weak reactions with some of the human phages. Only four strains were non-typable with any phage.

The association of lysis between phages on these 397 strains is shown in Fig. 1, where only strong reactions were considered. This figure consists of a series of bars corresponding to the percentage of strains lysed by one phage (shown in the left-hand column) which were also lysed by the other phage (shown in the horizontal row at the top). Of the group I phages, 52 and 52A usually gave lysis only with that of 29 and 80. The new phage 13 appeared to belong to this group, although its lytic spectrum (see Table 5) was similar to the other bovine phages. Only phage 71 appeared useful among the group II phages. Group III phages gave rise to many different patterns of lysis.

Lysis by phage 42D was observed in staphylococci from 8 of 52 control and survey herds and from 13 of 280 isolates from bulk milk. This phage often gave lysis with phages of lytic group III, especially phages 42E and 47.

Phage 81 acted with the group III phages, except in the seven strains of phage type 80/81 mentioned above.

Phages 101, 102, 107, 108 behaved generally as group III phages and phage 102 gave lysis with phage 42 D more than with any of the group III phages. Phage 110 was broader in its action and lysed in the main strains which could not be allotted

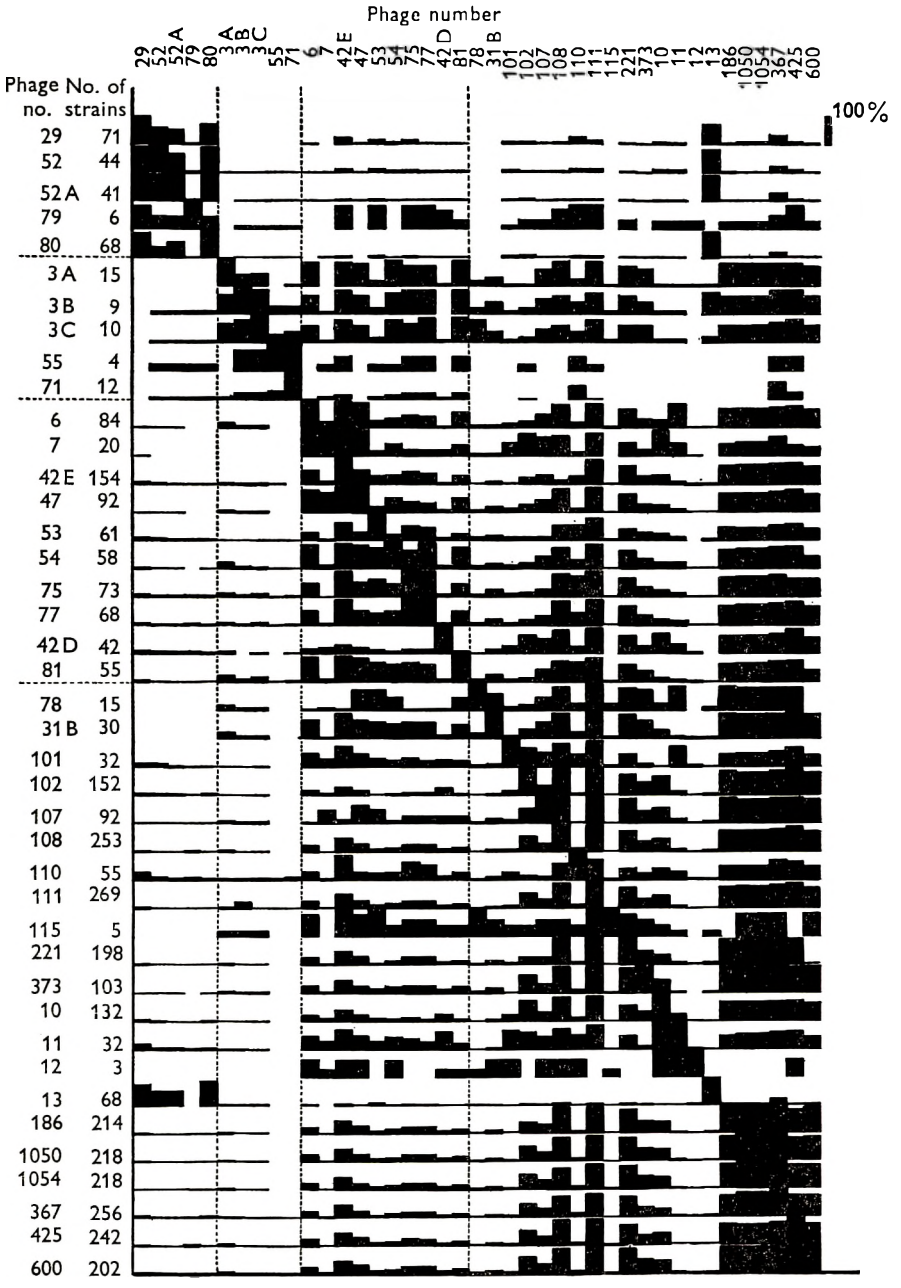

Fig. 1. Patterns of lysis of 397 strains of *Staphylococcus aureus* by 41 phages. Each horizontal line shows the pattern of lysis of those strains which gave a strong reaction with the phage shown in the column on the extreme left. The height of each bar indicates the percentage of these strains which were also lysed by the phage shown in the top line. The strains were derived from 328 herds, and included only one member of each phage group from each herd.

to any lytic group. It was associated particularly with phage 42E, and also phage 29. Phage 111 was the most active of all, and lysed 70% of all strains typed.

Many of the phages isolated in Queensland were actively lytic, and phages 1050 and 1054 gave identical patterns of lysis. Although phages 367, 425 and 600 appear similar in Fig. 1, they each gave lysis alone on a number of strains.

Table 5 shows the lytic spectra of four of the new phages (nos. 13, 367, 425 and 600) on a set of test strains, some recommended by Blair & Williams (1961) for testing the specificity of the human typing phages, and the rest from the bovine set of Davidson (1961). The serological groups of the four phages, which were determined by Dr P. M. Rountree of the Royal Prince Alfred Hospital, Sydney, are also given in the table.

Table 5. *Lytic spectrum and serological group of new phages*

	Phage			
	13	367	425	600
Serological group	B	B	B	A
Propagating or test strain:				
52	—	—	—	—
52A/79	—	1	—	—
53	—	1	—	—
54	—	1	—	2
29	—	—	—	—
42C	—	—	—	—
42E	—	3	—	2
47	—	—	—	—
2009	—	3	2	—
8719	—	—	—	—
31B	—	—	—	—
42D	—	3	1	1
71	—	—	—	—
75	—	—	—	—
77	—	1	—	—
80	—	—	—	—
78	—	1	—	—
101	3	3	3	4
102	—	—	3	2
105/107	3	4	4	3
108	3	4	4	4
110	1	4	2	4
111	3	4	4	4
115	—	—	—	—

DISCUSSION

The human set of phages was extremely useful on its own to type bovine strains, and the lytic groups were distinguished here as recognized in the typing of human staphylococci. This is with the exception of group II, where lysis was uncommon and strains lysed by these phages were usually unable to be classified into a specific lytic group.

The position of phage 42D is rather curious. Early workers found that bovine

staphylococci were commonly lysed by this phage (Macdonald, 1946; Smith, 1948*b*), and for this and other reasons strains of this phage type were considered of probable animal origin. The phage was separated from phages of lytic group III and is the only member of lytic group IV in the human phage set. In the present study, the endemic strains in the first two herds examined were lysed by this phage and this accounts for the large number of group IV and miscellaneous strains in Table 1. When observations were extended to other herds, staphylococci lysed by this phage were not common. The phages of bovine origin often lysed strains along with phages of group III, and phage 42D was usually associated with the bovine phages. When weak reactions with phage 42D were considered, in 39 strains this was associated with strong lysis by the bovine phages, in 53 strains with both these and group III, but never with phages of group III and without the bovine phages. Thus in some respects it was a 'link' between group III phages and those of bovine origin. However, Fig. 1 shows that phage 42D did tend to form a group of its own and in this respect it was not greatly dissimilar to phage 53, which, although usually reacting with other phages of group III, also reacted separately from others of the group. Perhaps the bovine group, along with phage 42D, could be considered a subgroup of group III.

The 42 phages were also used to type 96 strains from other animals. Only two of 78 dog strains were typed. One belonged to lytic group III, and a strain of identical pattern was isolated from the nose of the owner. The other was lysed by phage 187. This is the only strain ever lysed by this phage in this laboratory. Four of nine strains from cats were lysed, with the following patterns: 53, 53/10, 81, 6/75/77/10. The remaining strains, from horses, fowls and sheep were not lysed by any phage.

No set criteria can be laid down to define a suitable phage, and the selection of phages suitable for the typing of bovine strains is largely a matter of trial and error. However, it is important to consider the purpose of phage typing when a selection of phages is to be made. In studies on human strains its greatest use is in small epidemiological investigations, especially in hospitals. Here it is immaterial which phages are used to distinguish between cultures from related sources. It has been most useful in some epidemiological investigations (Parker & Kennedy, 1949; Sompolinsky, Hermann, Oeding & Rippon, 1957). From this type of investigation, it became clear that strains of particular importance appeared, and it was desirable to gain information of their distribution within and between countries. It is then essential that the phages used be similar. Such investigations resulted in the selection of the human set as a basis for the typing of human strains, with any hospital laboratory using additional phages if required.

In the selection of a set of phages for typing bovine staphylococci, therefore, the nature of the information required should be considered. Apart from research, at the present time there seems little likelihood of typing being of any practical value in investigating herd outbreaks where, as pointed out above, the particular phages used are unimportant.

On the broader scale, however, we need to know much more of the distribution of patterns among the dairy cattle population, and it is highly desirable that we

can compare these patterns with those in other countries. A suitable basic set for typing bovine strains should be able to satisfy the following criteria, in order of importance:

(1) It should bear as close a resemblance as practicable to the human set so that comparisons can be made.

(2) A high proportion of strains should be typed. However, provided the proportion of *herds* in which non-typable strains are predominant is low, it is not an ideal that every strain should be typed.

(3) Each phage should have a limited host range so that overlapping of phage patterns is minimized.

(4) Enough phages should be present in each lytic group to distinguish types within groups as clearly as possible. These types should be consistent and not too complex.

(5) It is not practicable to use more than 25 phages on one carpeted plate, so the set should be less than this to allow for additions.

A set of phages was selected from the 42 which was considered suitable for use on bovine strains in Queensland and probably Australia. The set consists of the following phages:

Group I	29, 80, 13
Group II	71
Group III	6, 7, 42E, 47, 53, 77, 31B
Group IV	42D
Others	81
	101, 102, 107, 110, 600, 367, 425.

Phage 80 was selected from the '52/52A/80 complex' of Rountree & Asheshov (1961). This along with phages 29 and 13 were the most useful of group I. As phage 81 is also included, the recognition of phage type 80/81, an important human pathogen, should be possible. Phage 71 was the obvious choice from group II, but more difficulty was found with group III, and perhaps one or more of 6, 7, 47 and 77 could be deleted if necessary.

This set of 20 phages, on the series of 1404 strains of staphylococci, would not have typed a further 35 strains, making a total of 129 or 9·2% of non-typable strains. Since this investigation the set has been used to type a large number of bovine staphylococci from all states of Australia, and has so far proved satisfactory.

Lysogeny among staphylococci would appear to be a universal phenomenon and it is clear that many phages thus isolated have too broad an action to be useful in phage-typing. It is of interest to note that the phages isolated here which showed the least action, were from cross-spotting of cultures from widely different sources. This approach should perhaps be followed in isolating new phages, rather than obtaining them from local strains where prophage immunity may be significant.

SUMMARY

Preliminary typing of 1820 strains of *Staph. aureus* from 51 herds with the human set of phages showed that non-typable strains were common (31·4%).

Other phages were investigated and 1404 strains from 328 herds were typed with 42 phages; these included the human phage set, phages from Weybridge and phages isolated in Brisbane. The following 20 phages were selected as suitable for typing bovine strains of *Staph. aureus* in Australia: 29, 80, 13, 71, 6, 7, 42E, 47, 53, 77, 31B, 42D, 81, 101, 102, 107, 110, 600, 367, 425. Phages 13, 367, 425, 600 were from Brisbane and 101, 102, 107 and 110 were from Weybridge.

The author is indebted to Miss Y. Battey, Department of Health, Brisbane, for the preliminary typing with the human phage set; to Dr Phyllis Rountree, Royal Prince Alfred Hospital, Sydney, for her advice and encouragement and for grouping the new phages serologically; to Mr I. Davidson, Central Veterinary Laboratory, Weybridge, U.K., who kindly supplied his phages; to Miss J. McCall for skilled technical assistance; and to Professor J. Francis for his support.

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Salmonella in meat imported from South American countries

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INTRODUCTION

The increasing demand for animal proteins in Europe has led to a considerable rise in the importation of meat from South American countries. This importation is not without risk from the public health point of view, since English and Dutch research workers have already pointed out that this meat may be contaminated to a high degree with salmonellas (Hobbs & Wilson, 1959; Hobbs, 1964; Eenink, 1966). The purpose of this investigation was to gain a deeper insight into the degree of contamination in various types of imported meat.

MATERIALS AND METHODS

Chilled and frozen horsemeat and beef, either carcass meat or boneless, is imported into the Netherlands from South American countries. The investigation reported here concerned chilled carcass horsemeat, frozen carcass and boneless horsemeat and boneless beef. The samples of frozen meat were sampled at twenty-five import stations by the local meat inspectors. The samples of chilled horsemeat originated from only one import station. The investigation was carried out from June 1965 to February 1966.

Method of sampling

To obtain samples of chilled horsemeat, whole limbs were jointed at the knee. Each lower joint was packed separately in plastic bags and transported directly to the laboratory. In addition, a certain number of joints were thoroughly swabbed with moistened cotton-wool pads in order to examine a greater surface area.

Samples of frozen horsemeat and beef, boneless and carcass, were sawn out or cut out of the blocks of meat using disinfected instruments. The size of the samples was approximately $20 \times 10 \times 10$ cm. The samples were separately packed in plastic bags and transported to the laboratory. In a few instances the samples were not packed separately and the results of examinations on these samples will be reported separately.

Method of examination

The samples of boneless and carcass meat were placed on a sterile tray and, with sterile instruments, 50 cm.^{2} of the superficial layers of connective tissue or of the meat were removed. These severed pieces were used to determine external

contamination. The cut surface of the original sample was then flamed and sampled once more. Scissors and forceps were used for chilled meat and a drill for frozen meat to obtain samples of approximately 20 g. When the size of a sample was too small for proper internal examination it was examined twice externally. This means that instead of 50 cm^2 an area of 100 cm^2 of the surface was examined for *Salmonella*.

The salmonella examination was carried out by enrichment of 50 $cm.^2$ of the surface of each sample or 20 g. of the interior of the sample in the media of Müller–Kauffmann and Osborne–Stokes (Osborne & Stokes, 1955). After 24 and 72 hr. incubation at 37° C. the cultures were streaked on brilliant green-phenol red agar plates (diameter 14 cm.) which were examined after 18–24 hr. incubation at 37° C. (Guinée & Kampelmacher, 1962).

RESULTS

From nine different batches of chilled carcass horsemeat, all originating from one single firm in Argentina, 108 samples (12 per batch) were examined over a period of 2 months. Moreover, complete joints of five of these batches were examined externally by swabbing. No salmonellas were isolated from these samples.

Table 1. Salmonella *in frozen horse meat*

Country of Origin	No. of samples examined	Samples positive		Samples examined once externally and once internally				Samples examined twice externally		
					Positive				Positive	
		No.	%	No.	Ext.	Int.	Both	No.	One	Both
Boneless meat										
Argentina	76	41	54	58	13	0	17	18	8	3
Brazil	8	3	37	7	2	0	0	1	1	0
Uruguay	212	126	59	119	47	1	20	93	34	24
Total	296	170	57	184	62	1	37	112	43	27
Carcass meat										
Argentina	264	28	10	241	20	3	1	23	3	1
Brazil	17	0	0	16	0	0	0	1	0	0
Uruguay	80	28	35	76	25	0	3	4	0	0
Total	361	56	15	333	45	3	4	28	3	1
Not specified										
Argentina	143	52	36	97	13	2	5	46	18	14
General total	800	278	34·7	614	172 (28·0 %)			186	106 (56·9 %)	

A total of 296 samples of frozen boneless and 361 samples of frozen carcass horsemeat originating from Argentina, Brazil and Uruguay were examined. It was not possible to ascertain the numbers of different batches from which these samples originated. In addition 143 samples of frozen horsemeat from Argentina

were examined; it was not possible to ascertain whether the meat was boneless or not. The results of these examinations are summarized in Table 1.

Of frozen boneless beef originating from Argentina, Brazil and Uruguay, 751

Table 2. Salmonella *in frozen beef (boneless)*

Country of origin	No. of samples examined	Samples positive		Samples examined once externally and once internally		
		No.	%	External only positive	Internal only positive	Both positive
Argentina	563	59	10	54	0	5
Brazil	28	0	0	0	0	0
Uruguay	160	42	26	28	4	10
Total	751	101	13	82	4	15

Table 3. *Frequency of* Salmonella *serotypes isolated from frozen horsemeat and beef*

	Frozen horse meat	Beef
S. anatum	133	33
S. minnesota	115	8
S. oranienburg	83	4
S. typhi murium	36	27
S. good	33	1
S. newport	25	35
S. raus	19	1
S. montevideo	16	6
S. haelsingborg	12	0
S. derby	12	8
S. saint paul	11	3
S. muenchen	8	0
S. siegburg	8	0
S. vaertan	8	0
S. meleagridis	6	2
S. oldenburg	2	0
S. stanley	2	0
S. dublin	1	0
S. san diego	1	3
S. java	1	0
S. bovis morbificans	1	4
S. haifa	1	0
S. cerro	1	0
S. bredeney	0	3
S. panama	0	2
S. give	0	1
S. infantis	0	1
S. livingstone	0	1

samples were examined. Again it was impossible to ascertain the numbers of different batches from which the samples were taken. The results of these examinations are summarized in Table 2.

Table 3 shows the frequency of *Salmonella* serotypes isolated from frozen horsemeat and beef.

The results of the examinations of a number of samples which were not packed separately are given in Table 4.

Table 4. *Prevalence of* Salmonella *in the samples of frozen boneless horsemeat which were not packed separately and were externally examined twice*

Country of origin	Number of samples examined	1 × positive	2 × positive
Argentina	43	5	38
	Frequency of types		
S. typhi murium	37	*S. muenchen*	3
S. minnesota	23	*S. san diego*	3
S. anatum	18	*S. meleagridis*	3
S. oranienburg	18	*S. montevideo*	1
S. gaminara	5	*S. saint paul*	1
S. newport	3	*S. cerro*	1

DISCUSSION

It is clear from the results that the method of examination strongly influences the number of positive results obtained. For example, the percentage of positive results among samples of frozen boneless and carcass horsemeat varied from 28·0 to 56·9, depending on whether one or two external examinations were carried out. Comparisons therefore between different countries of origin or between the different conditions of importation is only possible when the method of examination is uniform throughout. When a comparison is made between the samples which were examined once externally and once internally then the absence of salmonellas from the samples of chilled horsemeat is striking. All these samples originated from the same slaughterhouse in Argentina.

Out of 184 samples of frozen boneless horsemeat 99 (53 %) were found to be positive externally, while of 333 samples of carcass meat only 49 (14 %) were positive externally. There was also a striking difference between the number of samples positive internally, 38 (20 %) of frozen boneless compared with 7 (2 %) of carcass samples of horsemeat. It is clear from these results that contamination in the slaughterhouse is an important factor.

The pattern of contamination of the samples of frozen boneless beef agreed broadly with that found for positive samples of carcass horsemeat: 12 % were externally and 2 % were internally positive.

When the countries of origin were considered Uruguay showed the highest percentage of contaminated samples. The results shown in Table 4 are of special interest; they indicate that unhygienic handling of the meat (in this case contact between parts of different samples) can increase the percentage of contamination up to 100 when two external samples are examined.

The percentage of contaminated samples of Argentina frozen boneless meat agrees well with the results obtained by Hobbs (1964). The percentages of positive

results found by this author for the period 1961–63 were 43 for horsemeat, 54 for horse offal and 15 for beef. Furthermore, the *Salmonella* types found in our study agree well with those found in England. Three of the four most frequently isolated types from horsemeat were the same. For beef the three most frequently isolated types were the same.

Considering the ten types most frequently isolated from minced meat in the Netherlands (Report 1966) and the serotypes listed in Table 3, it is apparent that six of the ten are the same as the seven most frequently isolated types from South American frozen meat. This agreement between the types found leads to the suspicion that meat imported from South American countries is not only used in the meat industry but also by butchers in the preparation of minced meat.

It is evident from the investigations by Hobbs (1964) as well as from those reported here, that slaughterhouse practice plays a very important role in establishing these high rates of contamination. From the public health point of view, we are dealing with a difficult situation, since measures to prevent contamination have to be taken in the country of origin.

SUMMARY

Salmonellas were isolated from 278 of 800 (34·7 %) samples of frozen carcass or boneless horsemeat from South America and from 101 of 751 (13·5 %) samples of frozen boneless beef. However, the percentage of contamination detected depends to a large degree on the method of examination. Contamination was mostly external, a fact which points to slaughterhouse contamination. Boneless meat was contaminated to a higher degree than carcass meat.

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Serological studies on infections by respiratory viruses of the inhabitants of Tristan da Cunha

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The volcanic island of Tristan da Cunha, in the South Atlantic, has been inhabited continuously since 1816. It is a barren place remote from seaports and regular shipping routes and is infrequently visited (see Samuels, 1963). In 1961 the volcano became active and the island was therefore evacuated and the 264 inhabitants were brought by ship directly to England with a brief stop at Cape Town on the way. Their origins and their state of health on arrival here have been described elsewhere (Samuels, 1963; Lewis, 1963). They lived for three months in wartime barracks in Surrey and then they were accommodated by families in separate well-built houses in Calshot, Hants (Black, Thacker, Lewis & Thould, 1963).

While on the island some adults had an influenza-like illness in 1950 and there was a severe outbreak of influenza in 1954. After a ship had called there was sometimes a wave of colds (Woolley, 1963). It was known that in 1955 the islanders of all ages had almost no antibodies against poliovirus types 1 and 2, although they had antibodies against type 3. They were therefore given live poliovirus vaccine in 1961 (Gear, personal communication). There had been only one recorded epidemic of measles in which both children and adults were affected. Chicken pox-zoster and infectious hepatitis had also occurred in recent years (Taylor-Robinson & Tyrrell, 1963). It was, nevertheless, thought that these islanders had been to a large extent isolated from contact with respiratory and other viruses and that they might therefore suffer excessively from respiratory diseases as soon as they reached the outside world; this was supported by the fact that although they stopped for only a few days in Cape Town most of them had colds by the time they arrived in Britain.

A few sera taken before the evacuation had been studied previously and had been found to contain antibodies against parainfluenza virus, respiratory syncytial virus and rhinoviruses (Taylor-Robinson & Tyrrell, 1963; J. E. Doggett, personal communication); as the sera lacked antibodies against Asian influenza virus the islanders were given influenza vaccine after they arrived. They continued to suffer from frequent colds and respiratory illnesses during their first months in England and three old people died of pneumonia, the lesions of which on pathological examination resembled virus pneumonia more than a bacterial disease.

It was not possible to do thorough virus isolation studies, but samples of the sera of many islanders were available and these are listed below. We used these sera to determine what the response to influenza vaccination had been and, as far as possible, what respiratory viruses had infected the islanders after they left Cape Town and during their stay in Britain. The results of these studies are reported below, but as individual records of the illnesses from which the islanders suffered in Britain were not available it has been impossible to correlate individual serological results with individual respiratory tract illnesses.

The main recorded events of the islander's lives which seem to bear on this paper are as follows:

1954	Epidemic of clinical influenza.	
1961	10 October	Volcano erupted. Islanders taken by liner to Cape Town.
	17 October	Islanders reached Cape Town, and visited the City and were bled.
	21 October	Islanders left Cape Town.
	3 November	Reached England and were taken to Pendell Camp.
	21 December	Received influenza vaccine.
1962	6 January	Bled for the first time in England.
	23 January	Moved from barracks to single family, permanent houses at Calshot.
1963	30 April and 10 May	Bled for the second time in England.

MATERIALS AND METHODS

Sera

There were no sera from islanders under 8 years of age, but half or more of all the other age groups were available. The sera were all separated aseptically and stored frozen. The sera were usually diluted 1/5 in cholera filtrate (Phillips) and incubated overnight at 37° and for 30 min. at 56°, and stored at −20° (Tyrrell & Horsfall, 1952). Some sera were diluted in saline and stored at −20° C. There was no apparent loss of antibody titre after repeated freezing and thawing, except on one occasion with sera which had been diluted in saline. Comparisons of the titres of successive sera from each individual were always made on sera which had been diluted at the same time and frozen and thawed in the same way, and control positive human sera were always included in each test and showed no significant changes in the sensitivity of the tests.

Viruses

The following strains of influenza virus were used:

Influenza A Swine—Shope strain.
Influenza A0—MEL.
Influenza A1—Kunz; A/Eng/1/51 and A/Eng/1/53.
Influenza A2—A/Pakistan/1/57.
Influenza B—Lee 1940; B/Eng/10/54; B/Eng/939/59.

All these viruses were grown in the allantoic cavity of embryonated eggs.

Influenza C—strain 1233 was usually used as amniotic fluid.

Parainfluenza virus type 1—Cop 222 or C39 were used; the former was grown in monkey kidney cells and the latter in the allantoic cavity of chick embryos.

Parainfluenza virus type 2—a pool of egg-adapted virus supplied by Dr De Meio, National Drug Company, Philadelphia.

Parainfluenza virus type 3—bovine strain 33 propagated in calf kidney cells.

Reoviruses—type 1 and type 2, prototype strains ECHO 10 and Sue.

Echovirus 11—U virus strain of Philipson; this and the reoviruses were grown in rhesus-monkey cells.

Coxsackievirus A21—a strain isolated in England and usually propagated in human embryo-kidney cells.

All viruses were stored frozen at $-70°$ except for some pools of reovirus which were stored at $-20°$ C.

Titrations

We had barely 0·5 ml. of most of the sera and we wished to perform numerous titrations. Haemagglutination-inhibition tests were therefore performed by the micromethod of Takátsy (Sever, 1962), using 0·025 ml. volumes. Four haemagglutinating (HA) units of virus were allowed to react for 1 hr. at room temperature with serial dilutions of serum before adding 0·025 ml. of 1 % group 0 human red cells. Chicken cells, 0·5 %, were used with parainfluenza type 2. Complete inhibition of haemagglutination was taken as the end-point. The diluent was isotonic sodium chloride buffered at pH 7·1 with 0·01 M sodium phosphate buffer.

Table 1. *Comparison of the efficiency of haemagglutination inhibition and neutralization tests on paired sera from eleven islanders in detecting rising titres of antibody against three influenza A viruses*

		Results of test by	
Test virus	Serum	Neutralization	Haemagglutination-inhibition
MEL A0	57	< 5/10	< 10/40
	62	< 5/80	< 10/40
	191	10/40	< 10/160
	218	< 5/20	< 10/80
A Swine	30	< 5/> 80	< 10/40
	49	< 5/> 80	< 10/160
	131	< 5/> 80	< 10/120
	140	10/80	10/60
Kunz A1 47	218	10/320	5/120
	31	5/60	10/40
	30	< 5/> 320	10/160

It was found that the micromethod gave very reproducible results, and the results agreed well with those of neutralization tests with influenza virus (Table 1). In spite of economy in the use of the sera many became exhausted during the

course of the investigation and therefore it was not possible to test all specimens with all viruses. At the end of the experiments reported here attempts were made to perform complement-fixation tests, but too many of the remaining sera were anticomplementary for useful results to be obtained.

RESULTS

Influenza viruses

Soon after the epidemics of influenza in 1954, antibodies against influenza A1 and B had been found in the sera of islanders (M. A. Westwood & J. H. S. Gear, personal communication quoted Taylor-Robinson & Tyrrell, 1963). However,

Table 2. *Influenza antibody status on leaving Tristan da Cunha*

		Percentage of sera collected in Cape Town which contained antibody against indicated serotype								
Sera collected	No. of sera tested	A swine	A0 '33	A1 '47	A1 '51	A1 '53	A2 '57	B '40	B '42	B '59
October 1961	103–129	0	0	0	25	9	0	0	2	0

Inhibiting haemagglutination at the initial dilution of 1/10 or greater.

Table 3. *Initial response to influenza vaccine containing A 2 and B' 59 strain*

		Percentage of islanders with rising titres against indicated virus							
Sera collected	No. of pairs tested*	A swine	A0 '33	A1 '47	A1 '51	A1 '53	A2 '57	B '40	B '59
October 1961 and January 1962	132–138	43	52	80	95	92	83	17	85

* According to records nine Islanders who were supposed to have received vaccine did not get it. However, as one of these had an antibody rise against Influenza A2 all the serum pairs have been included in this analysis.

by October 1961 only low titres could be detected (Table 2) in about a quarter of the sera collected in Cape Town. In December 1961 the islanders received at 1 week intervals two injections of 1 ml. of a formalin-inactivated vaccine containing 7500 HA units of the strain of A2/Singapore/1/59 and 5000 units of B/England/59/59 suspended in saline (manufactured by Pfizer Ltd., Sandwich, Kent). The islanders were bled thereafter in December 1961 and January 1962, and most of them showed antibody against viruses of the type present in the vaccine. However, in addition, most of them developed antibodies against viruses of the A1 types which had been prevalent at the time of the epidemics on the island (Table 3). Furthermore, many developed antibodies against the A Swine, A0 and B Lee viruses which had been prevalent before the recorded outbreaks of influenza.

There was no evidence that Influenza A or B were active in the south of England at that time and attempts to isolate influenza virus from a number of islanders

suffering from influenza-like illnesses were unsuccessful (G. Cook, personal communication).

It was possible that the rises in the titre of antibody against 'old' influenza strains were due to the recall of antibody which had been induced by infection during unrecorded or unrecognized epidemics on the island before 1950. A previous serological survey (see Taylor-Robinson & Tyrrell, 1963) had shown that there were antibodies to 'earlier' serotypes in sera collected in 1955, but only in subjects

Table 4. *Changes in titres of antibody against influenza virus in 76 pairs of sera collected after vaccination and before departure from Britain*

		Percentage of subjects with indicated finding					
Sera collected		A swine	A0 '33	A1 '47	A2 '57	B '40	B '59
January 1962	Antibody present	17	61	89	83	40	85
May 1963	Antibody present	3	3	72	100	83	99
	Rises*	1	0	0	51	13	30
	Falls*	7	26	57	9	3	0

* Rising or falling titres of fourfold or greater.

Table 5. *Relationship between antibody titres after vaccination and further rises in titre against influenza A and B viruses presumably due to natural infection*

Virus used in test	Antibody titre after vaccination	No. of islanders	Percentage of islanders with rising titres against
Influenza B '59	< 10	11	91
	10	6	83
	20	12	67
	40	40	8
	80 or 160	6	0
Influenza A2 '57	< 10	14	93
	10	2	(100)
	20	11	100
	40	23	17
	80 or 160	26	0

over 16 years of age at that time, and it was thought then that there might have been an epidemic of influenza A in 1934 or of B in 1938. If that were so it was expected that antibody following vaccination in 1961 would be recalled only in those who were alive at the time of prevalence of the earlier serotypes, i.e. those over 15 years in the case of A viruses and probably over 30 years in the case of A Swine viruses. The antibody titres were therefore arranged according to the ages of the donors, and the results are shown in Fig. 1. These graphs demonstrate clearly that at all ages the antibody response to the A1 strain A/Eng/1/51 was as great as that to the A2 serotype given in the vaccine (Fig. 1*a*, *b*). No specimens were obtained from children under eight who might not have been sensitized to the

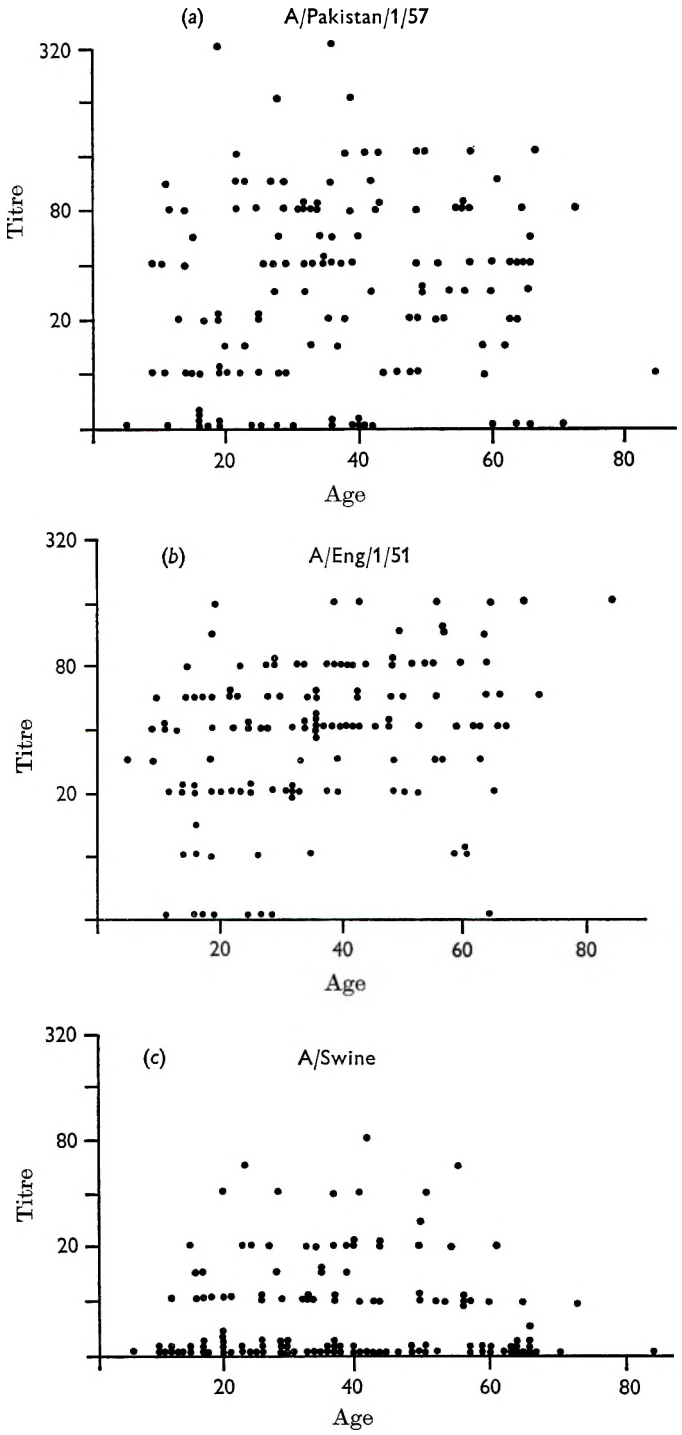

Fig. 1. Haemagglutination-inhibiting antibody titres against influenza viruses in the sera of islanders of various ages bled in January 1962 after being vaccinated with influenza A 2/ Sing/1/57 and influenza B/Eng/59/59. The distribution of antibody titres against para-influenza viruses was rather similar to that of antibodies against A/Eng/1/51.

A strain, but it is quite clear that the antibodies to the A Swine and A strains were found in subjects of all ages, although the titre and the frequency of antibody were less against the 'older' strain A 0, and least against the 'oldest' strain, A Swine (Fig. 1 c). The best explanation of these findings is that the islanders had been sensitized to a wide range of antigens and produced a fairly 'broad' antibody response. The fact that they reacted in the same way irrespective of age suggests that they had all had the same antigenic experience in the past; this is consistent both with the reports that epidemic influenza appeared on the island only in recent years and that antibodies were found in most sera collected in 1955.

Further sera were collected in 1963 and where possible these were compared with the sera collected immediately after vaccination. The results are summarized in Table 4, which shows that while antibody against 'old' strains of influenza A declined, further rises in antibody titres against influenza A 2 and B occurred. These were observed only in subjects with low antibody titres after vaccination (Table 5), and as both viruses were known to be prevalent in Britain between 1961 and 1963, it is assumed that they are due to natural infections with influenza A 2 and B viruses. Antibody to a titre of about 1/40 was apparently able to prevent infection with these viruses. Antibody titres against the A 1 1947, A 0 and A Swine viruses, on the other hand, declined and there was no evidence that the antibodies against the A 1 1947 or earlier strains were boosted by natural infections with the recent A 2 strains, although they had been boosted by vaccination. There were, however, some increases in titre against influenza B strains, possibly because the antigens are more closely related to current antigen than those of the A 2 serotypes and influenza A 1 strains. In the end, virtually all the islanders acquired antibody against the current serotypes of influenza A and B.

Table 6. *Age distribution of* 76 *subjects tested for rising titres against parainfluenza* 1 *and* 3 *viruses*

Age (years)	No. of subjects	Age (years)	No. of subjects
0–5	1	31–40	19
6–10	3	41–50	12
11–15	6	51–60	6
16–20	7	61–70	9
21–30	11	Over 70	2

Antibody responses to other respiratory viruses

The sera were next titrated against other haemagglutinating viruses which are certainly or possibly associated with upper respiratory tract disease. The age distribution of the donors of the paired sera tested appears in Table 6 because of the surprising finding that most adults were apparently infected with parainfluenza 3 virus, which usually affects infants and children in this country. The results of the serological tests are shown in Table 7, together with some results on unpaired sera. In many tests it was possible to titrate paired sera from about one-third of the islanders, but owing to shortage of sera or antigens the numbers

Table 7. *Presence of H.I. antibodies against other respiratory viruses and changes in titre*

Sera collected		Percentage of subjects showing indicated findings								
		Parainfluenza							Reovirus	
		1	2	3	Mumps	Influenza C	Coxsackie A21	Echovirus 11	1	2
October 1961	Antibody present	23 (128)	50 (12)	48 (128)	—	69 (68)	13 (86)	—	—	—
January 1962	Antibody present	80 (76)	100 (20)	99 (76)	85 (35)	91 (75)	14 (76)	12 (137)	0 (76)	0 (76)
May 1963	Antibody present	72 (76)	100 (15)	100 (76)	83 (36)	54 (72)	25 (76)	—	0 (76)	3 (76)
First period (October 1961–January 1962)	Rises*	83 (80)	50 (12)	95 (80)	—	10 (68)	7 (86)	1 (137)	—	—
Second period (January 1961–May 1963)	Rises	1 (76)	20 (15)	1 (76)	3 (35)	0 (71)	5 (76)	—	0 (76)	0 (76)
Second period	Falls	72	20	26	0	18	3	—	0	0

Number of sera or serum pairs shown in parentheses.

* Fourfold or greater rises or falls.

were reduced in some of the later tests. Nevertheless, certain quite striking facts emerged. For instance, in the first period of study, almost all the islanders showed antibody rises against parainfluenza 3, many against parainfluenza 1 and some against type 2. During the second period there were many falls in antibody against type 3 and both rises and falls against type 2. There were also antibody rises against influenza C in the first period and some falls in the second. Mumps antibody was detected although the disease had not been reported on the Island (Samuels, 1963). Some rising titres were also detected against coxsackievirus A 21 in both periods of study. On the other hand, antibodies against the reoviruses were infrequent and no rising titres were detected. Since very different frequencies of antibody rises were noted using different viruses as antigens it is unlikely that the results were due to loss of antibody during the storage of the sera collected in Cape Town; these sera were in any case handled with great care and stored almost as long as the next sera by the time some of the tests were made.

We wondered if these antibody results were not specific, but it has been shown that the HI titres as measured in this laboratory give results which agree well with those of specific homologous neutralizing antibody to parainfluenza 1, 2 and 3 (Taylor-Robinson, 1965; Taylor-Robinson & Bynoe, 1963), reoviruses (Brown & Taylor-Robinson, 1966), echovirus 11 (Buckland, Bynoe, Philipson & Tyrrell, 1959) and coxsackievirus A 21 (Buckland, Bynoe & Tyrrell, 1965).

Table 8. *Relationship between antibody titre in first serum of a pair and the presence of a rising titre*

Virus used in test	Antibody titre of first specimen	No. of sera	Percentage of paired sera showing rising titre
Parainfluenza 2	< 10	6	100
	10	7	63
	20	6	17
	40	8	0
Influenza C	< 10	29	24
	10	40	18
	20	29	3
	40	2	0
Coxsackie A 21	< 10	67	15
	10	8	0
	20	4	0
	40	3	0

The apparent correlations between low antibody titre and the appearance of a rise are statistically significant.

It was, of course, possible that some of the antibody responses were due to cross-reactions between the antigens; for instance, it is likely that the antibody responses to parainfluenza 1 were due to infection with the type 3 virus or vice versa. However, for both the periods of study it was found that, as in the case of the influenza antibodies, rising titres against parainfluenza 2, influenza C and cox-

sackievirus A 21 occurred almost entirely in persons without detectable antibody or with only low titres (Table 8); there is other evidence that antibody rises against coxsackievirus A 21 are not induced by several other biologically related picornaviruses (Doggett, Buckland & Tyrrell, 1964). As the proportion of antibody responses to the other viruses, such as parainfluenza 3 or reoviruses, was either almost 100 % or nil, it was not possible in these cases to detect a correlation between antibody titre and the occurrence of a subsequent rise. We nevertheless believe that the rises observed were due to infection with viruses closely related antigenically to those used in the tests.

In populous areas such as Britain and the U.S.A. antibody rises against parainfluenza viruses or isolations of these agents are only common among infants and young children with respiratory disease (Chanock & Parrott, 1965) and the titres of normal adults persist at a high level (Taylor-Robinson, 1965); it is therefore remarkable that in this investigation rising titres of antibody were found in subjects of all ages. The antibody distribution resembled the uniform response of islanders of all ages to influenza vaccine which is seen in Fig. 1*a* and *b*. In one respect, however, the pattern of antibody responses did resemble that seen in populous areas. It can be seen from Table 7 that infections seemed to occur earlier with parainfluenza virus types 3 and 1 and later with type 2, and this is exactly the pattern seen in children in the first few years of life in Britain (Stark, Heath & Peto, 1964). It also looks as though coxsackievirus A 21 spread inefficiently and slowly even under barrack conditions and this is consistent with what is known of its behaviour in civilians and servicemen in Britain (McDonald, Miller, Zuckerman & Pereira, 1962; Pereira & Pereira, 1959). It might therefore be said that, like infants who have lost their maternal antibody, these islanders acted as a sentinel population; they picked up those viruses which were circulating in the area, and picked up most frequently those which are known to be readily transmitted among children.

Later during their stay the antibody titres of the islanders against parainfluenza and influenza C viruses declined (Table 7) and few further rises were detected, which suggests that these viruses did not attack the community again.

Relation between blood groups and virus infection

It was shown by McDonald & Zuckerman (1962) that in the R.A.F. relatively more men with blood group O than with group A were admitted to hospital with serologically recognized influenza A 2 infections. However, they had no means of determining whether subjects of known antibody status were more likely to become infected with an influenza virus if they belonged to blood group O, or whether the antibody response on which they diagnosed the disease was more likely to occur in subjects of group A than of group O. As the Tristan islanders were of a different racial origin and as we knew their antibody status while in Britain, we wished to correlate their antibody responses with their blood groups in order to extend the observations of McDonald & Zuckerman. The blood groups of the islanders were kindly supplied to us by Dr A. E. Mourant and our results were analysed in order to see whether there was any correlation between the blood

groups and the presence of antibody on arrival in Britain or the acquisition of antibody afterwards.

As shown in Table 9, on arrival in Britain more of the islanders who had antibody against influenza A1/51 belonged to group A than to group O and the difference was statistically significant. This finding might have been due to subjects of group A being more susceptible to infection with influenza A1 virus when the virus was epidemic on the island. The possibility was also considered that the differences might be due to the production of differing amounts of antibody to

Table 9. *Frequency of antibodies against influenza* A/Eng/1/51 *in sera collected on arriving in Cape Town from persons of various blood groups*

	No. of persons in blood group				
Antibody	A	O	B	AB	All groups
Present	19	1	2	2	24
Absent	23	27	3	2	55
Total	42	28	5	4	79

Table 10. *Frequency of rising titres of antibody in subjects with antibody titres of* 20 *or less*

	Number of pairs of sera showing antibody rises—classified by blood group				
Virus used	A	O	B	AB	All groups
Influenza B/Eng/59	*13/17	6/9	2/2	2/2	23/30
Influenza C	4/12	3/14	0/1	0/2	7/29
Coxsackie A21	5/30	3/30	1/5	1/1	10/66
Totals	22/59	12/53	3/8	3/5	40/125
	37%	23%			32%

* Numerator = number of pairs in which a rising titre was observed. Denominator = number of pairs tested.

the same infection; however, further analysis showed that when exposed to an identical 'new' antigenic stimulus, namely vaccination with influenza A2, the frequency and height of the antibody response was the same in subjects of both blood groups to all the influenza A and B strains. An attempt was therefore made to detect the postulated difference in susceptibility to infection by studying a small number of subjects who were without protective levels of antibody at either the first or second bleeding. It was shown (Table 10) that a relatively higher proportion of those in Group A than those in group O developed antibody, presumably due to infection with one of the three viruses used in the test. The difference was almost significant at the level $P = 0{\cdot}05$. Antibody against influenza C was not apparently related to blood group and the titre of antibody against influenza A and B was not apparently related to the presence of M and N blood groups. It seemed that the ABO blood group was related in some way to resistance to infection, but nevertheless the presence of antibody was a much more important factor.

DISCUSSION

Recent serological surveys from this laboratory have shown that a high proportion of sera collected from normal subjects living in cities or in remote and isolated villages in many parts of the world, contain antibodies against the common respiratory viruses, such as influenza, parainfluenza, respiratory syncytial virus, reoviruses and rhinoviruses (Taylor-Robinson, 1965; Doggett, 1965; Brown & Taylor-Robinson, 1966). This implies that all these viruses are able to spread in human communities in very varied conditions of climate, housing and standard of living. The extreme degree of physical isolation of Tristan da Cunha was, however, apparently sufficient to reduce detectably the degree to which the inhabitants were exposed to viruses and immunized against them.

The antibody responses of the group to influenza vaccination were remarkable, but not unique for islanders have been found in the Pacific who had apparently never been exposed to infection with influenza viruses (Brown, Gajdusek & Morris, 1966), but it must still be very rare to find a whole community which has apparently been exposed to only two epidemics of influenza virus and to administer a uniform antigenic stimulus to all of them. The Eskimos studied by Reinhard & Gerloff (1960) and Reinhard (1962) were probably less isolated than the inhabitants of Tristan da Cunha. The Tristan islanders of all ages apparently responded mainly to the viruses prevalent when the epidemics occurred on the island and this can be interpreted as a striking natural experiment in confirmation of the ideas of Davenport and his colleagues (Davenport & Hennessey, 1957). They believe that the broadness and altered specificity of the response of older subjects to vaccination with monospecific vaccines is due to their 'experience' of more and different influenza antigens and not to any hypothetical ageing process. On the other hand, it should be noted that although the islanders were apparently exposed to only two serotypes of influenza A virus there were many antibody responses to viruses only distantly related to these. It may be significant that these antibodies declined rather rapidly and did not seem to be recalled by natural infection. It is likely therefore that they were not really comparable with those found in the sera of old subjects who have not been vaccinated and carry antibody against viruses to which they were probably exposed only in early life. Experiments in rabbits show that the dosage of virus given to an animal already sensitized to a distantly related virus may decide whether the animal produces more of an older type of antibody or gives a primary type response against the 'new' antigen (Fazekas de St Groth & Webster, 1966). In the case of the islanders it is, however, theoretically possible that after a long period without reinforcement their immunological 'memory' might fade. If it had been possible to apply the technique of Drescher (Drescher, Hennessey & Davenport, 1962) or to perform adsorption experiments, one might have demonstrated whether the antibodies were directed primarily against the old viruses or the newer ones.

There is little need to comment further on the results with the other respiratory viruses. The remains of the sera were in too poor condition to do successful complement-fixation tests otherwise we might have tried to detect infection with

adenovirus or R.S. virus. It is likely that the influenza vaccine gave some useful protection and if an effective vaccine against parainfluenza viruses had been available the islanders might have been protected against these viruses. The data suggest that, with the technique used, antibody to a titre of 1/40 or more protected almost all subjects against influenza infections, and this titre apparently protected volunteers against infection with influenza A 2 and B viruses (Bell *et al.* 1957; Buckland, Bynoe & Tyrrell, unpublished); a titre of 1/20 might have protected them against parainfluenza 2 infection (Table 8). The results draw attention again to the mysteries of the clinical significance, if any, of antibodies against influenza C virus, an agent which is almost never isolated in Britain, although it seems to be associated with respiratory disease. Antibodies against reoviruses are found in sera of British residents, but these viruses are not known to be associated with disease here, they are rarely isolated and were not apparently transmitted to the islanders.

We were rather surprised by the results of our analysis of the relation between infection and blood groups which seem to show that subjects with blood group A are more likely to be infected with influenza and some other viruses than those of group O. This is the opposite of what we expected on the basis of the work of McDonald & Zuckerman (1962). There seem to be two possible explanations. One is that there may be two opposing effects of the blood groups—the possession of group A for example may render a subject both more likely to be infected by influenza A 2, and less likely to be made ill by it. This is difficult to believe. The other alternative is that this is not an effect of blood groups at all, but of some other factor which, in the case of natives of Britain, is linked with blood groups O and A in one way, and in the islanders in the opposite way. This resistance appeared to be effective against a number of viruses in our study although only the combined data approached statistical significance. Sheddon & Potter (1964) also found a correlation between the frequency of antibodies against 'epidemic' adenoviruses and the blood groups of children in Sheffield. On the other hand, Downie *et al.* (1965) could find no support for a suggestion that the frequency or severity of smallpox in India might be correlated with the possession of A, B and O blood groups.

SUMMARY

Sera were obtained from about half of the Tristan da Cunha islanders on reaching Cape Town in October 1961, a few months after reaching Britain and before returning to the Island in 1963.

After vaccination against current influenza A 2 and B serotypes antibodies were evoked against earlier serotypes, in particular against influenza A 1 of 1951 with which the islanders had almost certainly been infected. Those who failed to develop antibody against A 2 and B after vaccination developed it later presumably owing to natural infection.

Almost all islanders developed antibodies against parainfluenza 3 and 1 and some against parainfluenza 2 and coxsackie A 21 viruses. There were no antibody rises against reoviruses.

There was some evidence that islanders belonging to blood group A were more susceptible to infection with influenza and coxsackie viruses than were those belonging to blood group O.

We are most grateful to the Medical Research Council Working Party on Tristan da Cunha and its Secretary, Dr H. E. Lewis, and also to Dr J. H. S. Gear of the South African Institute for Medical Research who collected the sera in South Africa, and basic data regarding the islanders. We also thank Dr A. E. Mourant for generously supplying us with full details of the results of blood-grouping tests on the islanders.

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Investigations on the incidence of rinderpest virus infection in game animals of N. Tanganyika and S. Kenya 1960/63

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It is generally considered that all animals of the Order Artiodactyla are susceptible to infection with rinderpest virus, although convincing evidence is lacking for many species (Scott, 1959). There is also a consensus of opinion in veterinary circles that game animals can play a most important role in maintaining the virus in East Africa, even in the absence of susceptible cattle, and furthermore that they have often been responsible for rapid and extensive spread of the disease (see, for example, Lowe, 1942; Thomas & Reid, 1944; Wilde, 1953; Branagan & Hammond, 1965). In addition, from the point of view of the wildlife conservationist or biologist, rinderpest is undoubtedly the most important infectious disease of wild ruminants because of its potential killing power (Blayney Percival, 1918; Simon, 1962), and the impossibility of prophylaxis.

With these considerations in mind all available game animal sera were examined for rinderpest-neutralizing antibody beginning in 1960 and, with the exception of the year 1964, continuing to date. The sera were predominantly from wildebeest, the reservoir host of malignant catarrhal fever virus (Plowright, 1965) but smaller numbers of other species were obtained later. Practical difficulties in obtaining sera unfortunately excluded the African buffalo from this list, although it is undoubtedly most important in the epizootiology of rinderpest. This paper deals with the results obtained in the initial 3 years of the investigation, during the first half of which rinderpest infection was still frequent in some species. A brief account of some of the findings to 1962 was given in an earlier paper (Plowright, 1963*a*).

MATERIALS AND METHODS

Sera

Blood from animals which had been killed by shooting was obtained by severing the neck or other large vessels, whilst animals immobilized by drugs were bled from the jugular vein. The blood was usually allowed to clot and stand overnight at environmental temperature before removing serum for clarification by centrifugation. Serum samples were then stored at 4° C., usually preserved by the addition of a drop of chloroform, and later forwarded on ice; after arrival at the laboratory they were stored until required at -20 to $-25°$ C. and then thawed and inactivated at 56° C. for 30 min.

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Rinderpest neutralization tests

Undiluted serum was tested against $10^{1.6}$ to $10^{2.8}$ TCD 50 of culture-adapted virus of the Kabete 'O' strain (Plowright, 1962). Two tubes were used per sample and the protection of one or both of them against the cytopathic effects of the virus was considered to indicate a significant level of neutralizing activity. The great majority of positive samples were then titrated for neutralizing activity using $10^{1.8}$ to $10^{2.6}$ TCD 50 of virus per tube and an identical technique to that already described (Plowright, 1962). A standard immune cattle serum, not inactivated, was included in each test as a check on the sensitivity.

Log_{10} SN 50 titres were calculated by the method of Thompson (1947); where serum at a final dilution of 1/2 only protected one or two of a total of five tubes, titres were expressed nominally as $10^{0.1}$ and $10^{6.2}$ respectively, for inclusion in calculations of mean titres. Titres were ordinarily written as the reciprocal of the $\log_{10}$ SN 50 end-point.

Animal populations

The populations of blue wildebeest (*Connochaetes taurinus*, Burchell; syn. *Gorgon taurinus taurinus*, Burchell) were in general the same as those already described (Plowright, 1965). However, in the light of more recent studies (Estes, 1966; Watson, 1967) it is apparent that the wildebeest occurring in areas to the west of the Great Rift Valley can be subdivided into several ecologically distinct groups.

By far the largest population is the Serengeti migrant group, which numbers at present 320,000–380,000 head (Watson, 1967), moving in vast hordes on to the central plains and towards Ol Balbal during the wet season (December to June) and dispersing mainly to the west in the remaining dry season (Watson, 1965, 1966; Figure 1). At the western end of the Serengeti National Park is a small group of about 4000 head, resident in the Kirawira area; they disperse during the dry season with migrant animals. In the Ngorongoro Crater there are about 10,000–15,000 resident wildebeest, of which about 35% leave the caldera for the Ol Balbal Plains during the wet season (Estes, 1966; Watson, 1967); whilst there they may intermingle with migrant herds but they return to the Crater in the dry season. A further resident group is found in the Mara-Loita Plains area of Kenya and these animals may establish contact with Serengeti migrants as a result of their southward movement during the dry season; they number about 15,000–20,000 head.

The age of wildebeest was estimated from their dentition and general development, aided by the fact that the great majority of calves are born usually within a period of 6–8 weeks extending from February to April in the Kajiado area and from December to February in areas to the west of the Rift Valley. In ageing very young calves consideration was also given to the state of the umbilical cord, hooves, central incisors and horn buds.

In the Ngorongoro Conservation Area during the period early February, 1962 to June of the same year efforts were made to obtain samples from as many dam-

calf pairs as possible. This was usually accomplished by shooting the calf first and then the dam as she turned back towards her offspring. It was thus possible to collect materials for a study of the relationship between serum antibodies in the dam and the calf during the first 4–5 months of life of the latter.

Sera from eland (*Taurotragus oryx* Pallas), Thompson's gazelle (*Gazella thomsoni* Gunther), Grant's gazelle (*Gazella granti* Brooke), and Coke's hartebeest (*Alcelaphus buselaphus cokii* Gunther) were nearly all obtained within the Ngorongoro Conservation area, a proportion of them within the Crater itself. Additional sera from Thompson's gazelle were obtained from the Naivasha area of Kenya, where rinderpest is not enzootic.

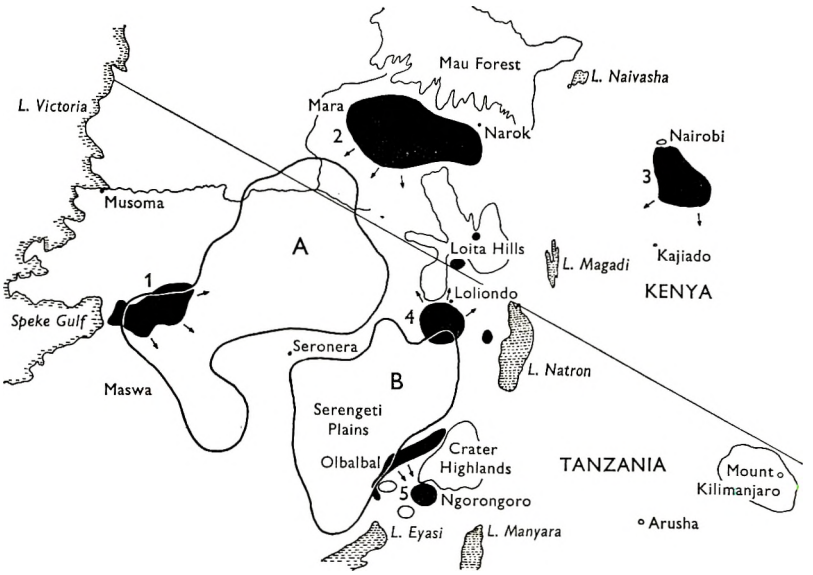

Fig. 1. The main wildebeest populations of East Africa (with acknowledgements to Watson, 1967). A. Dry season dispersal area of the main Serengeti migrant population (July to November). B. Wet season concentration area of Serengeti migrant wildebeest: (1) Kirawira resident group; (2) Mara National Park: Loita Plains group; (3) Kajiado: Nairobi National Park group; (4) Loliondo resident group; (5) Ngorongoro resident group. Arrows indicate the direction of dry-season dispersal.

RESULTS

(1). *Confirmation of rinderpest in the Serengeti National Park—October/November 1960*

In October 1960 an outbreak of severe disease with considerable mortality occurred in 'yearling' wildebeest in the Serengeti National Park; when observed on 6 to 8 November the mortality had already declined considerably, but virtually all 'yearling' wildebeest exhibited a harsh, discoloured pelage with faeces drying on and adherent to the tail and perineal region. There had also been a considerable loss of condition. Five affected animals, whose age was estimated to be 7–9 months, were killed and attempts were made to isolate virus from their spleens and blood by inoculating 5% (w/v) suspensions of the former or leucocyte fractions from

10 ml. of the latter into culture of primary calf kidney cells; the techniques have already been described in detail (Plowright & Ferris, 1962). No cytopathogenic agent was detected.

A sixth yearling animal was found dead, the carcass being partially eaten by predators; its spleen also failed to yield virus but a positive result for rinderpest virus antigens was obtained in agar gel diffusion tests against hyperimmune rabbit serum (Scott & Brown, 1961*). In this animal the only lesion which was suggestive of rinderpest was epithelial necrosis and caseous deposits on the palatal tonsils; in the other five animals no abnormalities indicative of rinderpest infection were seen.

Sera from all six yearlings were tested for rinderpest neutralizing antibody and found to be positive; the log SN 50 titres in five animals which were killed, against $10^{2\cdot4}$ TCD 50 of virus per tube, ranged from 1·8 to $\geqslant$ 2·7, with a mean of at least 2·38, considerably higher than any mean titre subsequently recorded in any group of serologically positive wildebeest (*vide infra*). The high level of antibody also indicated that the five animals were in an advanced phase of convalescence, thus explaining the failure to recover virus.

(2). *The distribution of serologically-positive animals in Kenya wildebeest Kajiado game controlled area and Nairobi National Park*

A total of seventy samples was obtained between 1960 and 1963 and the results of screening tests for rinderpest antibody are given in Table 1.

Table 1. *Rinderpest antibody in Kenya wildebeest 1960–63 Kajiado–Nairobi National Park Areas*

	Year of sampling		
Year of birth	1960	1961	1963
⩽1957	2/9*	—	
1958	0/1	—	2/16
1959	0/3	—	
1960	0/4†	—	
1961	—	3/14‡	3/23§
1962	—	—	

* No. positive/no. tested.
† All calves 3/4 months old.
‡ All calves < 1 month old, of which 7 may not have received colostrum.
§ Titres 0·8, 0·8 and 'trace'.

In 1960 although two of nine adults were positive, no animal less than 3 years old had antibody. In 1963, however, there were three of twenty-three sera from immature animals, i.e. probably 1–2 years old, which did have antibody. The SN 50 titre of two of the positives, 0·8 against $10^{2\cdot4}$ TCD 50 of virus, was sufficiently high to indicate that no error had occurred in the preliminary screening test; in the third animal a titration revealed only a trace of antibody. The presence of anti-

* We are indebted to Dr G. R. Scott for carrying out these tests.

body in 3/14 young calves captured in 1961 was presumably a reflexion of successful colostral transmission, although all these animals had been abandoned by their dams very early in life (Plowright, 1965) and half of them did not have antibodies to malignant catarrhal fever virus (M.C.F.) although this was universally present in adults. These animals may not, therefore, have received a feed of colostrum.

In the aggregate, it can be seen that seven of fifty-two animals (13·3 %) which were one or more years old showed evidence of previous infection with rinderpest virus. One serum from an adult eland shot in the Kajiado area in 1963 also exhibited an SN 50 titre of 0·8 against $10^{2·2}$ TCD 50 of virus.

Table 2. *Rinderpest antibody in Kenya wildebeest; Mara–Loita Plains 1960*

Year of birth	No. positive/ no. tested	Remarks
⩽1957	12/13	—
1958	3/3	—
1959	5/6	All obtained in March 1960
1960*a*	3/4	Calves up to 3 months old
1960*b*	0/6	Calves 5–8 months old
Total	32	

Mara National Park and Loita Plains, 1960

Between March and September 1960, serum samples were collected from thirty-two wildebeest as shown in Table 2. It was evident in this region that antibody passed via the colostrum of immune dams to the majority of young calves but that it had declined to non-detectable levels in the 5–8 months age group. Of six yearling animals born in early 1959 and sampled in March 1960 there were five which already showed evidence of previous infection with rinderpest virus and a very large proportion (*ca.* 94 %) of the adult animals similarly possessed antibody.

The serological conversion of calves born in 1959 could well have been due to an extension of the rinderpest infection reported to have been present in wildebeest in the Mara area of Tanganyika (Thomas, 1960).

(3). *The distribution of serologically-positive animals in Tanganyika wildebeest Ol Balbal Plains, 1962/63*

The month of collection of serum samples and the results of tests for rinderpest antibody are given in Table 3 for 1962 and Table 4 for 1963.

The majority of samples were obtained during the months January to May when both resident and migratory groups of wildebeest were present but the latter were almost certainly in great predominance and the figures can probably be regarded as typical for the migrant Serengeti population.

Of animals born in or before 1959 seventy-one of 81 (87·7 %) possessed antibody, the proportion of positives in the 1960 and 1961 calf crops falling to 75·8 % and 67·1 % respectively. The rising immunity rate in animals born in earlier years indicated that some individuals which escaped previous annual episodes became

infected in later ones (Table 3). It was evident from figures obtained early in the year 1962 that many calves born one year previously had already been actively immunized; assuming that they had not lost protective levels of colostral antibody until August or September, 1961, the infection must have been widespread in these herds at some time between then and January 1962. No disease or mortality in Serengeti migrant animals was reported during this time, however, although rinderpest 'took a fairly heavy toll of yearling buffalo during October and December' around the Ngorongoro Crater (Kinloch, 1963) and it was stated that the disease was responsible for the usual number of deaths of yearling wildebeest and buffalo in the Narok District of Kenya (Anon, 1962).

Table 3. *Rinderpest antibody in Tanganyika wildebeest; Ol Balbal Plains 1962*

Year of birth	Month of Collection									Positive %
	Jan.	Feb.	Mar.	Apr.	May	July	Aug.	Sept.	Totals	
⩽ 1958	6/6*	15/20	5/5	6/6	3/3	—	1/2	1/2	38/44	86·4
1959	5/5	9/10	12/13	2/2	4/5	1/2	—	—	33/37	89·2
1960	7/9	5/9	6/8	—	4/4	3/3	—	—	25/33	75·8
1961	23/31	11/15	9/16	8/10	2/5	0/1	0/1	—	53/79	67·1
1962	—	14/20	17/17	8/10	5/9	1/7	2/7†	0/2	See text	—
Totals	51	74	59	28	26	13	10	4	265	

* No. positive/no. tested.

† Positives estimated to be 8 months old, with titres of 0·6 and 0·8 against $10^{2\cdot6}$ TCD 50 of virus. Probably derived from resident population.

Table 4. *Rinderpest antibody in Tanganyika wildebeest; Ol Balbal Plains 1963*

Year of birth	Month of Collection					Totals
	Jan.	Feb.	Mar.	Apr.	June	
1961	—	—	—	—	1/1	1/1
1962	1/6	2/3	0/4	0/2	3/6*	6/21
1963	—	—	—	—	0/1	0/1
Totals	6	3	4	2	8	23

* Probably derived from the resident population.

Two animals estimated as 8 months old were found to be positive in August 1962 and there can be little doubt from the titres recorded that antibody was acquired following active infection; the time of collection made it very probable that these animals were members of a resident herd forming part of the Ngorongoro group. The single positive obtained amongst seven animals in July (Table 3) could have been due to residual colostral antibody; the calf involved was only 5 months old and the titre was very low.

During 1963, six of twenty-one animals born in early 1962 were found to have been infected and of these four had a reasonably high antibody titre (0·8), the other two exhibiting only trace amounts (Table 4). The time of collection of positive samples made it impossible to decide whether the low rate of infection which occurred during 1962 affected both the static (Ngorongoro) and the migratory (Serengeti) herds.

The figures in Table 3 for calves estimated to be less than 5 months old will be discussed in section 4 of the results.

Ngorongoro Crater, 1961/63

Tables 5 and 6 give details for collections made during 1961/62 and 1963 respectively. Whilst 100 % of the wildebeest 4 years old or more possessed antibody, the proportion in 3-year animals (75 %) fell somewhat below that determined in the Serengeti area (89 %) but the number of samples was small. The

Table 5. *Rinderpest antibody in Tanganyika wildebeest; Ngorongoro Crater 1961/62*

Year of birth	Month of collection									Positive %
	Dec. 61	Jan. 62	Apr.	May	June	Aug.	Sept.	Oct.	Totals	
⩽ 1958	16/16*	—	—	—	—	—	3/3	—	19/19	100
1959	—	—	4/5	0/1	2/2	—	—	—	6/8	75
1960	—	—	—	—	2/2	0/1	—	—	2/3	66
1961	0/8	0/10	2/11	1/4	0/11	2/5	1/3	—	6/52	11·5
1962	—	—	7/8	2/4	1/5	3/11†	0/3	0/5	See text	—

* No. positive/no. tested.

† Positives estimated to be 9 months old with SN 50 titres of 0·8, 1·8 and 0·4 against $10^{2\cdot6}$ TCD 50 of virus.

Table 6. *Rinderpest antibody in Tanganyika wildebeest; Ngorongoro Crater 1963*

Year of birth	Month of Collection				Totals
	Jan.	Feb.	Mar.	Nov.	
1962	0/6	0/13	0/3	0/2	0/24
1963	—	—	—	0/26	0/26

difference between the 1961 calf crops in the two localities was, however, very striking. The number of serologically-positive animals in the Crater was 11 % of the total tested, compared with 67 % of the Ol Balbal herds. A very few of the 1962 calf crop had also been infected by August of that year, with serum titres sufficiently high in two of three instances (Table 5) to leave no doubt of recent active infection, comparable to that in animals shot in June on the Ol Balbal Plains. The collections during 1963 failed to reveal any positive animal in fifty tested, of which twenty-four were born in the 1962 calving season (Table 6). It must, therefore, be considered possible that the Crater animals positive in August 1962 became infected during a seasonal move to Ol Balbal grazings.

Kirawira-Ikoma area, 1962/63

A total of thirty-two samples was obtained between December 1962 and July 1963; the results of their examination are given in Table 7. Rinderpest antibodies were distributed in a manner comparable to that already found in the main Serengeti migrant population (Table 3), but no serological conversion had occurred in calves of the 1962 season.

Titration of antibody in wildebeest sera

The titre of neutralizing antibody in groups of adult ($\geqslant$ 3 years old), 2-year-old and yearling wildebeest was determined in order to find if there was a decline with age. As shown in Table 8, yearlings of the 1960 calving season did have a somewhat higher mean titre than older animals, but the variance was very high and the difference was not significant ($t = 0{\cdot}39$; $P > 0{\cdot}5$). If, however, the titres of sixteen yearling sera collected in January 1962 ($1{\cdot}73 \pm 0{\cdot}77$) were compared with eighteen obtained in February to April 1962 inclusive ($0{\cdot}72 \pm 0{\cdot}40$), then there was a highly significant difference ($t = 3{\cdot}99$, $P < 0{\cdot}001$). This, together with the high mean titre ($\geqslant 2{\cdot}38$) recorded in 5 yearlings recently recovered from clinically-apparent infection in 1960, suggests that in the majority of animals at least antibody titres may fall rapidly after infection to levels such as those recorded in 2-year-old animals.

Table 7. *Rinderpest antibody in Tanganyika wildebeest; Kirawira/Ikoma 1963*

Year of birth	No. positive/ no. tested	Positive %
$\leqslant$ 1959	9/12	75
1960	3/3	100
1961	2/4	50
1962	0/13	Nil
Total	32	

Table 8. *The titre of rinderpest-neutralizing antibody in wildebeest sera*

Age group	No. of samples	Mean titre* and standard deviation	Range	Remarks
Yearling	34	$1{\cdot}19 \pm 0{\cdot}781$	0·1–2·8	All from Ol Balbal in Jan.–Apr. 1961
2 years	23	$0{\cdot}96 \pm 0{\cdot}513$	0·1–1·8	—
$\geqslant$ 3 years	67	$0{\cdot}92 \pm 0{\cdot}513$	0·1–2·6	—

* Reciprocal of $\log_{10}$ SN 50 dilution against $10^{1{\cdot}8}$ to $10^{2{\cdot}6}$ TCD 50 of virus.

(4). *The transfer of rinderpest-neutralizing antibody from wildebeest cows to their calves*

In the case of Ol Balbal and Ngorongoro wildebeest it has already been shown that at least 88 or 100 % respectively of the adults were immune and some animals negative in screening tests may well have had very small quantities of antibody which could have been excreted at a higher titre in the colostrum, as occurs in other ruminants (Brambell, Hemmings & Henderson, 1951; Brown, 1958). It was to be expected, therefore, that at least about 90 % of wildebeest calves which suckled in the first 24 hr. of life would possess rinderpest-neutralizing antibody. Only two foetal sera were examined and, although the dams were positive, no neutralizing activity could be detected.

To investigate this aspect of rinderpest epizootiology in wildebeest, titrations were carried out on fifty pairs of sera obtained from dams and their offspring during the first 5 months of life; in addition, twenty-five other sera were examined

from isolated calves between the ages of 3 weeks and 7 months. Of these twenty-five isolated calves, seven which were 10 weeks of age or less all had antibody and were presumed to have come from serologically-positive dams. Above this age about 10 % of negative calves could theoretically have been born to mothers without antibody.

As shown in Table 9 all of twenty-four calves which were 6–8 weeks old did have neutralizing antibody but four estimated to be $\leqslant$ 1 week and two more thought to be 2 weeks old were completely negative. These six animals were referred to in detail in another paper dealing *inter alia* with the colostral transfer in wildebeest of antibody against M.C.F. virus (Plowright, 1967); this antibody also, though present to high titre in the dams (SN 50 $\geqslant$ 1·6), failed to be transferred to the same six calves. Since the failure of maternal antibody to pass to these animals was thought to be possibly associated with abnormal factors such as calf disturbance due to shooting in the herds or severe stress factors in the dams (Plowright, 1967), data for them have not been included in calculating the figures given in columns 3, 4 and 5 of Table 9.

Table 9. *The acquisition and decline of maternally-derived rinderpest antibody in wildebeest calves*

Age of calf (weeks)	No. with antibody	Mean titre*	Mean titre† of dams	No. of calves with titre $\geqslant$ dam
1	3/7	1·47‡	0·93 (3)§	3/3‡
2–4	5/7	1·32‡	0·87 (3)§	2/3‡
6	7/7	1·26	1·00 (6)	6/6
8	17/17	0·98	1·09 (11)	9/11
10	6/8	0·76	1·03 (8)	3/8
12–14	8/12	0·39	1·01 (8)	0/8
16	4/7	0·46	0·70 (4)	1/4
20	4/5	0·24	— (1)	0/1
24	0/2	0·0	—	—
28	0/3	0·0	—	—

* Titres expressed as reciprocal of $\log_{10}$ SN 50 dilution.
† Figures in brackets refer to numbers on which calculations are based.
‡ Omitting calves in which colostral transfer of antibody did not take place.
§ Omitting dams whose calves did not acquire any maternal antibody.

The mean figures given in Table 9 show that during the first month of life the titre of antibody in the young was about 0·5 log units or 3 times higher than that in their dams; by the 6th week the mean titre in the calves was still about twice that of the cows but in the 8th week it had fallen below the maternal level and thereafter decreased rapidly. Individual calves, as shown in column 5 of Table 9, nearly always had as much or more antibody than their dams during the first 6 weeks, but by the end of the 3rd month the titre in the calf was almost constantly exceeded by that of its mother.

Some calves which were presumed to have received antibody via the colostrum in the normal manner were becoming serologically negative by the 10th week of

life and by the 6th and 7th months all passively-acquired antibody had disappeared. The rate of decline of colostral antibody in wildebeest was calculated from the regression of mean titres in column 3 of Table 9 and the half-life was found to be 4·4 weeks. Since the maximum titre recorded in any calf was 2·0 it was estimated on this basis that all passively-acquired rinderpest antibody in wildebeest would have passed its extinction point in 29 weeks or about 7 months, which corresponds very well with the observed figures. It also means that wildebeest calves are very largely susceptible to rinderpest by September of the year of their birth.

(5). *The distribution of serologically-positive animals in other species*

Eland. The results for sera of forty-seven obtained in the Ngorongoro Conservation area are given in Table 10.

It was apparent that eland suffered widespread infection with rinderpest virus before May and June 1962 when the majority of samples were collected in that year. This was hardly surprising in the case of the older animals, since sick eland

Table 10. *The distribution of serologically-positive eland in the Ngorongoro Conservation Area*

	Year of sampling			
Year of birth	1962	1963	Totals	Positive %
⩽ 1958	9/9	5/6	14/15	93
1959	3/6	1/2	4/8	50
1960	6/8	1/2	7/10	70
1961	1/2*	1/6	1/6	16
1962	—	1/6*	—	—
Totals	25	22	—	—

* Probably passively-acquired antibody in animals 6 months and 4 months old respectively. These are excluded from totals in column 4.

were observed on the Ol Balbal Plains in 1958 and a strain of rinderpest virus was isolated from one affected bull (Robson, Arnold, Plowright & Scott, 1959). In the case of eland calves born in 1960, they exhibited serological conversion in approximately the same proportion as wildebeest sampled at Ol Balbal or in the Ngorongoro Crater. The eland born in 1961, however, showed a low incidence of past infection, comparable to that in the Ngorongoro wildebeest calves of the same year and, so far as can be judged from small samples, much less than that in migrant Serengeti wildebeest born in early 1961.

Titrations were performed on all positive eland sera and in twenty-three animals which, by virtue of their age, were considered to have been actively immunized the mean log titre was 1·32 with a standard deviation of 0·48 and range of 0·8–2·2. This was somewhat higher than the level in wildebeest (Table 8).

Thompson's and Grant's gazelle, 1962/63. The results for sera from animals of these two species are given in Table 11. All were obtained in the Ngorongoro Conservation area, either in the Crater or on the Ol Balbal Plains. A low rate of infection had apparently occurred in Thompson's gazelle with some serological

conversion probably taking place in the latter half of 1961 or early 1962. There was no evidence of past infection in Grant's gazelle.

The titre of antibody in serologically-positive Thompson's gazelle was probably lower than that in wildebeest; thus in five animals the titre against $10^{2.6}$ TCD50 of virus varied from a trace to 1·0, the mean log SN50 being 0·66.

Of ten samples from *G. thompsoni* obtained in the Naivasha area of Kenya, six were from adults but none had demonstrable neutralizing antibody.

Table 11. *Rinderpest antibody in gazelles of the Ngorongoro Conservation Area 1962/63*

	No. with antibody in age group					
Species	1–4 months	5–8 months	9–18 months	2 years	3 years or more	Totals
Thompson's* gazelle	0/2	0/11	2/19	3/23	—	55
Grant's gazelle	—	0/7	0/14	0/18		39

* All obtained in 1962.

Coke's hartebeest and topi. Only four serum samples were examined from hartebeest, these being from animals 1, 2 or 3 years old. All were obtained in the Ngorongoro Conservation area and were negatve in screening tests. Two sera from 3-year-old topi at Ikoma were also negative.

DISCUSSION

The wildebeest has a particular significance in the epizootiology of rinderpest in East Africa, primarily because it is present in very large numbers, the majority of which form big aggregations and migrate regularly over long distances in pursuit of grazing or water. The total number of wildebeest in the Serengeti, Mara and Loita Plains was estimated to be about 220,000 in May 1961 (Stewart & Talbot, 1962), whilst in May 1963 a photographic count revealed over 300,000 head on the Central Serengeti Plains alone (Owen, 1964). Such a prolific, gregarious and mobile species affords excellent opportunities for the maintenance and dissemination of a virus like that of rinderpest.

The Annual Reports of the Veterinary and Game Preservation Departments of Tanganyika reveal the following historical background for rinderpest infection of wildebeest. The disease was rumoured to be present in the western Serengeti in October 1930 (Hornby, 1931), but it was not until March 1933 that the disease in wildebeest was confirmed for the first time by subinoculation into cattle of material from 2-year-old wildebeest found near the Ngorongoro Crater; rinderpest infection of a mild type was simultaneously observed in cattle and sick eland were seen later (Cornell, 1934).

Even at this time rinderpest infection in game animals was reported to be an *annual* occurrence near the Ngorongoro Crater but it was also asserted that it did 'not appear to do much harm' (Teare, 1935). Nevertheless, a considerable rinder-

pest mortality in 7–8 months old wildebeest was reported in the Serengeti area in 1935 (Teare, 1936), thus pointing to previous immunization of older age groups in the migratory population.

During the rest of the prewar and war period (1936–45) the available reports do not make possible any reconstruction of the annual disease events in the wildebeest or other specific populations of the Serengeti and adjacent areas. However, deaths in *young* wildebeest were reported in the Musoma district and Northern Province in late 1946 (Reid, 1947), and outbreaks were recorded in 1947 affecting wildebeest and giraffe in the Maswa area (Moore, 1948). The disease in wildebeest was confirmed on the northern range in 1948 (Moore, 1949) and in the Serengeti in November and December 1949 (Burns, 1950); it was suspected in the Serengeti in 1951 (Swynnerton, 1953), confirmed in the Mara area in 1953 (Swynnerton, 1954) and suspected again in the Serengeti in 1954 (Dawe, 1955). In the last year it was confirmed in wildebeest, buffalo and impala to the west of the Serengeti, without apparently gaining access to the park (Swynnerton, 1955). Although no rinderpest was confirmed in Tanganyika game animals in 1955/56 (Swynnerton, 1957), in October 1957 large numbers of wildebeest, 1–2 years old, died in the Musoma and Maswa districts, the disease disappearing by the end of the year (Swynnerton, 1958; Branagan & Hammond, 1965).

In early 1958, rinderpest was again confirmed in yearling wildebeest in Musoma and North Mara to the west of the Serengeti, with no apparent spread to other species; at the same time in the Crater Highlands the disease affected warthogs, buffalo and eland in addition to wildebeest but it had died out by July (Swynnerton, 1959). In 1959, rinderpest was thought to have been present in the Mara area at the beginning of the year, sick animals were seen in the south-east Serengeti during March and a heavy mortality occurred in yearling wildebeest in the northern part of the Ngorongoro Conservation area in December (Thomas, 1960). Only minor outbreaks of rinderpest in unspecified game animals were recorded in 1960 near the Ngorongoro Crater and in wildebeest calves to the west of the Serengeti (Kinloch, 1961). As already noted, the only serious outbreak of rinderpest reported in 1961 by the Game Department, Tanganyika, was one affecting yearling buffalo around the Ngorongoro Crater in October and December (Kinloch, 1963).

In summary of the more recent events it may be said that clinical rinderpest was reported annually in the main wildebeest concentrations of Tanganyika from 1957 to 1960 inclusive, and that it persisted in an easily recognizable form in buffalo until at least 1961. Before 1957, the infrequency of reports cannot be said to indicate the absence of infection for prolonged periods; in fact deaths largely among yearling and 2-year-old wildebeest were a sure indication that widespread infection must have taken place every 1 or 2 years. Talbot & Talbot (1961) referred to a comparable *annual* mortality in yearling wildebeest of the Mara region of Kenya; this began in October or November and faded out during January, by which time 40 % of the total initial calf crop were said to have succumbed to the disease.

The serological results reported in this paper confirm that widespread infection

of yearling wildebeest with rinderpest virus occurred in the Mara–Loita Plains area of Kenya during 1959/60 and on the Serengeti Plains during late 1960. About 76 % of the yearlings in the Serengeti migrant population of late 1960 had probably been infected, as well as a similar proportion (66 %) in the Ngorongoro resident herds. By December 1961 about two-thirds (67 %) of the calves born early in that year to dams of the migrant population had become immunized, but the proportion in the Ngorongoro Crater herds was much lower (11·5 %). The serological conversion in this year was peculiar in that it had not been accompanied by any signs of disease or mortality.

About 29 % of the calves born early in 1962 showed evidence of past infection by mid-1963 on the Ol Balbal Plains (Table 4), but some conversion had probably taken place here by August 1962 (Table 3). No signs of disease were detected in the wildebeest population during 1962 and hence, for the second year, rinderpest infection occurred silently.

The significance of small numbers of apparently silent infections needs to be considered in the light of their undoubted occurrence in the Kajiado area of Kenya (Table 1). They were shown to have occurred there both before and since 1960, affecting no more than about 15 % of the total population; in this district no disease or mortality has been reported in wildebeest in recent years, but mild strains of rinderpest were isolated from cattle in several localities of Kenya Masailand in early 1961 (Plowright, 1963*b*), and the Kajiado wildebeest probably had opportunities to contact such sick cattle. In a similar way Masai cattle, though excluded from the Serengeti Park, share the grazing of the whole of the Ngorongoro Conservation area with the game population and this contact may have been responsible for the infections detected in Ol Balbal and possibly Ngorongoro wildebeest born in 1962. Additional evidence that these infections did, in fact, occur on a significant scale at Ol Balbal was obtained by sampling carried out in 1966 and reported in a companion paper (Taylor & Watson, 1967, personal communication).

The involvement of only a small part of a susceptible population may have a number of implications. For example, between-animal contacts may not be sufficiently close or frequent to ensure adequate exposure of a large proportion of them; with a virus which is probably spread largely by aerosols this could well be true, especially in a tropical country where inactivation of a highly labile agent by desiccation and ultraviolet light would be expected to occur very rapidly. Secondly, the strains of virus involved may not be very invasive for wildebeest or other game species; a lack of invasiveness is well recognized for some strains in experimental cattle (Cooper, 1932; Taylor *et al.* 1965). Invasiveness may not be related to virulence, since it appeared that about two-thirds of the Ol Balbal yearlings suffered infection with a non-virulent strain in 1961; the low rate of infection amongst 1961 calves in the Ngorongoro Crater (Table 5) suggested that the strain of virus which was epizootic in the population outside may not have gained access to this relatively isolated group and that the few serological positives could have been infected during seasonal migration to the Ol Balbal Plains.

Our results for eland sera showed that this species behaved very similarly to

wildebeest in that infection was widespread up to 1960/61 but has since become less frequent. However, the total population of this species in the Serengeti area is very much smaller (6400–9600 according to Stewart & Talbot, 1962) and even if between-herd contacts were adequate, it could hardly maintain rinderpest virus for any prolonged period. Infection of Thompson's gazelle was not infrequent, unless the small number of samples examined gave an inaccurate indication of the rate of infection in the whole of the Serengeti population; assuming that the annual infection rate was approximately the same as that found in the 9–18 months age group—about 10·5 %—then as many as 50,000 infections could have occurred in a year, if we accept the lower census figure of Stewart & Talbot (1962). By this reckoning Thompson's gazelle could contribute materially to the maintenance of the virus.

The decline in the rate of rinderpest infection in game animals in the Serengeti, Ngorongoro and contiguous areas during the years 1962/63 and the continuing absence of widespread infection up to 1967 (Taylor & Watson, personal communication) is no reason for assuming that new and catastrophic invasions of the disease could not occur again. It appears that, for the first time since 1930, no strain of rinderpest virus is present which spreads readily in the most abundant and susceptible species; this is probably due in large measure to the efforts of the Veterinary Departments, which since 1950 have immunized as many as possible of the susceptible cattle in areas where cattle-game contacts are important (Branagan & Hammond, 1965). During and before 1961, in spite of prolonged efforts to immunize all susceptible cattle, it must be concluded that strains of virus persisted which spread readily in susceptible wildebeest, eland and probably also buffaloes and other species. These wild animals, in fact, may have been largely responsible for the maintenance of the virus at that time, whereas in recent years they have played a restricted role. If another strain of rinderpest virus, highly invasive for the predominant species, were to be introduced from outside, or were to emerge, following mutation or otherwise, from an existing undetected focus, then the results could hardly be less than spectacular.

SUMMARY

The incidence of rinderpest infection in game animals in selected localities of South Kenya and North Tanganyika was studied during the period 1960 to 1963. Serum samples from 590 wildebeest (*Connochaetes taurinus*), 48 eland (*Taurotragus oryx*), 65 Thompson's gazelle (*Gazella thompsoni*) and 39 Grant's gazelle (*Gazella granti*) were tested for rinderpest neutralizing antibody.

Rinderpest infection was shown to have been very frequent in yearling wildebeest in the Mara area of Kenya in 1959/60, in the Serengeti National Park of Tanganyika in late 1960 and also in the Serengeti, and some adjacent areas, during the latter half of 1961. In the Ngorongoro Crater in 1961 infection was far less widespread, with only 11 % of the yearlings acquiring antibody, compared to 67 % in the Serengeti. The infections in 1959 and 1960 were clinical epizootics, accompanied by a considerable mortality, whereas no overt disease was reported in the course of 1961. Eland were affected in a similar manner to wildebeest up to

1960 but only a low rate of serological conversion was demonstrated in 1961. Adult Thompson's gazelle showed a low rate (*ca.* 12%) of infection but no antibody was detected in Grant's gazelle.

Only a small proportion of the wildebeest calves born in early 1962 acquired antibody by mid-1963 and this was due, at least in part, to infection late in 1962; it was not clear, unfortunately, whether the positive animals belonged entirely to resident, as opposed to migratory, groups. No clinical signs or mortality were reported in this year.

A low incidence of rinderpest infection in wildebeest was also demonstrated both before and after 1960 in the Kajiado district of Kenya, where disease of game has not been reported in recent years. It is possible that the positive animals, as also the 1962 cases in Tanganyika, acquired the virus from low-grade infections of cattle.

The transmission of rinderpest antibody from wildebeest dam to calf, presumably via the colostrum, was demonstrated regularly, except in six calves about 1–2 weeks old. No completely satisfactory explanation was obtained for their failure to acquire passive antibody but it may have been due to abnormal disturbance in the herds, associated with the shooting. The antibody titres in calves were initially higher than those in the serum of their dams but by the end of the 3rd month this position had been reversed. Individual calves became serologically negative from about the 10th week of life and all were devoid of antibody by the 6th to 7th month. The half-life of passively-acquired antibody was 4·4 weeks.

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Heat resistance of spores of *Clostridium welchii**

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INTRODUCTION

Extensive investigations have been undertaken by British scientists in order to determine the epidemiology of *Clostridium welchii* food poisoning (Hobbs *et al.* 1953; Hobbs, 1965). According to these workers the organisms responsible for this type of food poisoning are atypical strains of *Cl. welchii* type A, differing from other members of the type A group by the production of low levels of alpha toxin (lecithinase), little or no theta toxin production (haemolysin), formation of heat-resistant spores (100° C. for 1 hr. or more), and by antigenic constituents which allow them to be grouped into thirteen provisional serotypes.

Hall, Angelotti, Lewis & Foter (1963) working at the Robert A. Taft Sanitary Engineering Center, in the United States, examined 83 strains of *Cl. welchii* from a wide variety of sources for their serological relationships, sporulation, heat-resistance of spores, and their haemolytic activity on mammalian bloods. They found that the American food-poisoning strains have a wide variety of characteristics and concluded that *Cl. welchii* food-poisoning outbreaks in the United States were not restricted to strains meeting the criteria of classification described by the British workers and that the isolation of large numbers of any strain of this organism from an incriminated food must be considered as having a possible bearing on the etiology of the outbreak.

On the other hand, Sutton (1966*b*) investigated an outbreak of *Cl. welchii* food poisoning and isolated heat-resistant *Cl. welchii* from ten of the twelve persons attending a small gathering and from all seven who developed symptoms of food poisoning. In another study, Sutton (1966*a*) studied the incidence of heat-resistant *Cl. welchii* in selected classes of a rural population and found that the carrier rate for the general population was low (1·5–6·0 %), but persons associated with communal feeding and poor hygienic conditions had a much higher carrier rate (15·1–25 %). Sutton (1966*a*) suggested that heat-resistant spores of *Cl. welchii* withstood the cooking process, particularly if the portion of meat cooked is large and bulky, germinated, multiplied, and caused food poisoning upon ingestion by a susceptible person. It had been previously shown by Collee, Knowlden & Hobbs (1961) that heat-resistant *Cl. welchii* grows rapidly within a temperature range of 23–50° C.

The purpose of the present study was to examine the heat-resistance of the

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spores of *Cl. welchii* isolated from soil, faeces, and food poisoning outbreaks in order to determine if patterns of heat-resistance are related to the source of isolation

MATERIALS AND METHODS

Strains of Clostridium welchii

Eight strains of *Cl. welchii* type A were employed in this study. They were strain BP6K (Boyd, Logan & Tytell, 1948), source unknown; UM 115, isolated from soil at the University of Montana; F 106, isolated from a human faecal specimen; FH 153, isolated by Dr H. E. Hall, Robert A. Taft Sanitary Engineering Center, Cincinnati, from a faecal specimen from a food handler; A 91, isolated from a pathological specimen at the Cincinnati General Hospital and obtained from Dr H. E. Hall; A 48, recovered from chicken broth that caused human food poisoning by Dr L. S. McClung, Indiana University, Bloomington (McClung, 1945); Hobbs's serotype 2, National Collection of Type Cultures number 8238, isolated from boiled beef suspected to have caused food poisoning; Hobbs's serotype 3, National Collection of Type Cultures number 8239, isolated from boiled beef suspected to have caused food poisoning. The cultures were maintained in Bacto-Cooked Meat Medium (Difco). The stock cultures were routinely checked for purity using morphological, biochemical, and haemolytic criteria.

Preparation of spore suspensions

The method recently developed by Schneider, Grecz & Anellis, (1963) for the sporulation of *Clostridium botulinum* and *Cl. perfringens* (*welchii*) in dialysis sacs was used in the present study for the production of spores. The apparatus consisted essentially of a telescoped cellulose bag immersed into the sporulation medium in a large Pyrex culture tube (see schematic illustrations in the publication by Schneider *et al.*). This method was used successfully in producing spores of *Cl. welchii* in four laboratory media (Groom & Strong, 1966).

Cultures of *Cl. welchii* were transferred at 12 hr. intervals in 20 ml. of Bacto-Fluid Thioglycollate Medium (Difco) in order to obtain vigorous growth of the vegetative cells. Then 150 ml. of broth were inoculated and incubated for 20 hr. at 37° C. The cells were centrifuged, resuspended in physiological saline and recentrifuged three times, and then the packed cells were resuspended in 10 ml. of sterile physiological saline. The entire contents were placed in the sterile sporulation dialysis bag which was suspended in a modified Wagenaar and Dack medium (Schneider *et al.* 1963). This medium consisted of 5·0 % Trypticase, 1·0 % peptone proteose (Difco) and 0·5 % sodium thioglycollate (pH 7·2). The sporulation apparatus was incubated at 37° C. for 9 days. The spores were harvested according to the procedures described by Schneider *et al.* (1963) and washed three times with 50 ml. sterile Sorensen's phosphate buffer (M/15, pH 7·0). The method of Long & Williams (1958) was used to separate vegetative cells from the spores. The final suspension of spores, in 10 ml. Sorensen's buffer, was stored at 4° C. Immediately after the preparation of spores the spore suspensions were stained with malachite green and examined microscopically to determine the degree of sporulation achieved.

Heat resistance studies

Thermal death time determinations were made using the stock spore suspensions which were diluted (1/100) with sterile Sorensen's phosphate buffer. Twenty-nine ml. of phosphate buffer, pH 7·0, was added to each reaction tube. The reaction tube consisted of a long Pyrex screw-capped culture tube containing a small ($\frac{5}{16}$ in. diameter) teflon-coated bar magnet. The reaction tubes were placed in a 6×10 in. basket and the basket was completely immersed in water contained in a constant temperature water bath at 90° C ($\pm 0{\cdot}5$° C). A stirring apparatus was used to rotate a small propeller-type blade to which was attached a teflon-coated bar magnet. As the blade with the magnet rotated in the hot water it agitated the small magnets nearby in the reaction tubes, inducing convection and cavitation, thereby exposing the spores to a more uniform water temperature. One of the reaction tubes contained a thermometer to record internal temperature. The tubes were allowed to reach equilibrium temperature and 1 ml. of the spore suspension was added to the tubes. Exposure to heat was timed 10 min. after the spores were introduced to the reaction tubes. The initial 10 min. period was considered a pasteurization period as well as a period for heat activation of the spores.

One-tenth ml. of the heated spore suspension was removed by a sterile 1 ml. pipette at designated intervals of time and immediately mixed in a culture tube containing 9·9 ml. of 1 % peptone-water which was previously cooled in an ice-water bath. The cooled contents were plated upon recovery medium consisting of 37 g. Bacto-Beef Heart for Infusions (Difco), 29·8 g. Bacto-Fluid Thioglycollate Medium (Difco), 15 g. agar, and 1000 ml. distilled water. Triplicate plates were inoculated. In addition culture tubes containing 10 ml. of fluid thioglycollate medium were inoculated. The plates and tubes were incubated in an anaerobic jar (Case Anaero-Jar) evacuated and filled with nitrogen gas at 37° C. for 48 hr. Plate counts were made with the aid of a Quebec darkfield colony counter and tubes were examined for the presence of growth.

Treatment of data

A logarithmic order of death was assumed and the data were treated according to the methods of Stumbo, Murphy & Cochran (1950), using the formula $D = U/(\log a - \log b)$. Using this formula D-values were computed from the straight-line portions of the thermal death time curves, where U = time of heating (minutes), a = initial spore population, and b = number of spores surviving at the end of heating period U.

Survival curves of the heat-treated spores were prepared by averaging arithmetically the three individual plate counts for each sample removed and determining the percentage survival and plotting these values as a function of time (exposure to heat). The trend in the data was analysed using the least-squares method. Only the data from the linear portion of the survival curves were treated by this method.

RESULTS

The sporulation method employed resulted in the complete production of spores. No vegetative cells were observed when the spore suspensions were stained with malachite green and examined microscopically. The spores were stored at 4° C. for several months without any apparent change in the numbers of spores. This procedure for sporulation was consistently reproducible.

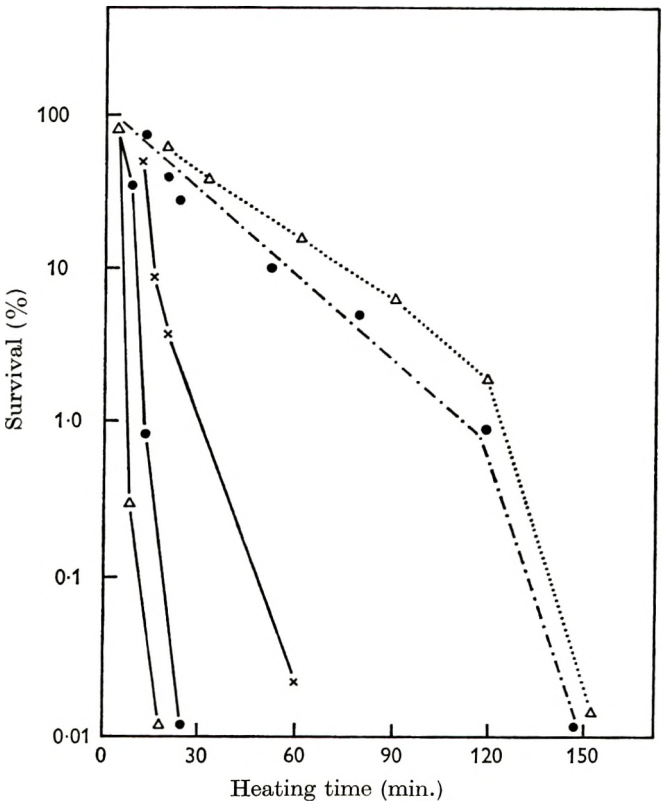

Fig. 1. Thermal survival curves for six strains of *Clostridium welchii*. △——△, UM 115; ●——●, BP6K, F 106; ×——×, FH 153; ●—·—●, Hobbs 3; △ - - - - △, Hobbs 2.

The effect of heat (90° C) on spores produced from different strains of *Cl. welchii* as a function of time is shown in Fig. 1. All strains exhibited a thermal death rate that was essentially exponential. Thermal destruction for strains UM 115, BP6K, F 106, and FH 153 was immediate and complete. On the other hand, strains Hobbs 2 and Hobbs 3 displayed considerable resistance to heat destruction. The heat-resistant spores could not be recovered after approximately 150 min. on the recovery plates but growth did occur in fluid thioglycollate medium. In some instances survivors grew in fluid thioglycollate medium after heating for 180 min. Weiss & Strong (1967) also reported similar findings.

D-values are presented in Table 1 for eight strains of *Cl. welchii*. The Hobbs strains displayed a significantly higher *D*-value. Weiss & Strong (1967) reported a

similar relationship, although their *D*-values were somewhat greater. A strain, UM 115, recently isolated from soil had the lowest *D*-value. This value was two-and-a-half orders below that of the food-poisoning strains.

The *D*-values were computed with the least-squares method and the trend of the data is shown in Table 2. When the values were plotted on graph paper a straight line trend was obtained.

Table 1. *D-values of* Clostridium welchii *spores heated at* 90° C

Strain of *Cl. welchii*	*D*-values
UM 115	0·015
BP6K	0·63
F 106	1·45
FH 153	2·28
A 48	7·07
A 91	4·93
Hobbs 3	6·05
Hobbs 2	8·71

Table 2. *Computed trend values of the D-values using the least-squares method*

Length of time heated at 90° C in minutes	Strain of *Cl. welchii*					
	A 48	A 91	F 106	FH 153	Hobbs 2	Hobbs 3
10	14·49	10·17	2·71	4·72	12·81	9·11
20	12·29	8·50	0·20	3·04	11·82	7·89
30	10·10	6·83	0	1·36	10·84	6·67
60	7·90	5·16	—	0	9·86	5·45
90	5·71	3·50	—	—	8·86	4·23
120	3·52	1·83	—	—	7·20	2·96
150	1·32	0·16	—	—	6·92	0

DISCUSSION

The heat-resistance of spores of *Cl. welchii* has been studied by numerous workers (Hobbs *et al.* 1935; Sutton, 1966*a*; Weiss & Strong, 1967; Hall *et al.* 1963). Since *Cl. welchii* is ubiquitous its heat sensitivity has been studied in many different parts of the world. It is apparent that heat-resistant and non-heat-resistant spores occur in nature (Collee *et al.* 1961). Hall & Angelotti (1962) reported that the spores of only 5 of 19 food-poisoning strains possessed heat resistance. The spores of *Cl. welchii* isolated by Dam-Mikkelsen, Petersen & Skovgaard (1962) from food-poisoning outbreaks in Denmark did not survive heating at 100° C. for 2 min. On the other hand Tong *et al.* (1962) investigated a food poisoning traced to turkey meat and isolated an aerobic sporulating bacillus which withstood boiling temperatures for 8 hr. Therefore, it appears that there is considerable variation in the reported findings with regard to the heat-resistance of spores of *Cl. welchii* isolated from food-poisoning incidents.

In the present study, although the number of strains studied was admittedly small, the organisms isolated from food-poisoning sources possessed a greater degree of heat-resistance than the strains isolated from other sources, particularly

soil. It has been suggested that some strains loose their heat resistance on repeated laboratory culture (McKillop, 1959). One strain of *Cl. welchii* which had its origin in a food-poisoning outbreak was readily killed at 80° C. for 10 min. (Canada, Strong & Scott, 1964). However, this strain had been carried on laboratory media for approximately 10 years.

It is conceivable that *Cl. welchii* isolated from food-poisoning outbreaks tend to exhibit heat-resistant spores (Brooks, Sterne & Warrack, 1957) primarily because the heat-sensitive spores were destroyed during the process of cooking the food. This could select the heat-resistant spores, although initially the spore population may have comprised both heat-resistant and heat-sensitive spores. It is well established that different strains of the same species may possess spores of unequal resistance to heat (Williams, 1929; Williams & Zimmerman, 1951; Mehl & Wynne, 1951; Briggs, 1966).

Nevertheless, it is reasonable to assume that the heat-resistant spores are primarily involved in *Cl. welchii* food poisoning because these are the spores that are more likely to resist destruction during the cooking or re-heating processes.

Currently, we are engaged in a study of the heat-resistance of spores isolated from various sources, namely, faeces, soil, food, and food-poisoning cases, and hope to correlate this property with lecithinase activity of these strains.

SUMMARY

Eight strains of *Cl. welchii* were studied for the heat-resistance of their spores. Spores of *Cl. welchii* isolated from food-poisoning cases had greater heat-resistance than strains isolated from soil or faeces. *D*-values and trend values were calculated from the thermal death curves.

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J. Hyg., Camb. (1967), **65**, 367
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With 1 *plate*

The Dip-Slide: a modified dip-inoculum transport medium for the laboratory diagnosis of infections of the urinary tract

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Determination of the concentration of bacteria in freshly voided urine can assist in the diagnosis of urinary tract infections, particularly by helping in the distinction between growth on culture which reflects infection and that which is the result of contamination during collection (Kass, 1956). Mackey & Sandys (1965, 1966) described a dip-inoculum transport medium which enabled such quantitative determinations to be made when rapid access to a laboratory is a problem, thus avoiding misleading impressions produced by the proliferation of organisms in urine after collection and before arrival at the laboratory. This paper describes a dip-slide which is based on Mackey and Sandys's idea of a solid transport medium inoculated by dipping and is an alternative to their nutrient medium filled spoon. The advantages of the dip-slide are that it is readily made from easily available materials, it is cheap, two different media can be inoculated simultaneously if desired, an area of 2 sq. in. of each medium is inoculated and colony counts are easy. Briefly, the dip-slide consists of a 3×1 in. microscope slide coated on one or both sides with nutrient medium for a 2 in. length; the slide is housed in a cylindrical aluminium container which grips it firmly with the uncoated portion of the slide at the capped end. In use, the slide is held by the uncoated portion, dipped in fresh urine and replaced in the container. On arrival at the laboratory, and a delay is of no consequence, the unopened container is incubated and the slide examined the following day. The number of colonies on the slide is a measure of the bacterial concentration in the fresh urine.

METHODS

Preparation of dip-slides

Microscope slides, 3×1 in., are sterilized in bulk. A suitable rack to hold the slides is described but the method could easily be varied (see Plate 1). A row of nine slides is clipped with $1\frac{1}{4}$ in. spring clips ('Bulldog' brand, M. Myers and Son Ltd.) to a 20×2 mm. Duralumin bar about 15 in. long fixed (with Evo-Stik) to large rubber bungs. The slides are handled with sterile forceps but the bar and clips are not sterile. Using a 10 ml. pipette, 1·5–2 ml. of molten agar medium at 50–55° C. is run on to each slide where it forms a layer over a 2 in. length of the slide. When

the medium has set, in 2 or 3 min., the whole bar with attached slides is turned over and a second medium run on to the other side of the slides. MacConkey's agar and nutrient agar are a suitable pair of media; this combination allows the growth of both Gram-negative rods and Gram-positive cocci which may cause urinary tract infections, proteus strains do not swarm on the MacConkey's agar and both media are commonly used for other purposes so that a special medium does not have to be prepared. But other media could be used.

Extruded aluminium screw-capped containers 27 × 75 mm. (The Metal Box Company Ltd.), commonly used in the Public Health Laboratory Service as faeces containers, are sterilized without a cardboard liner in the cap but with a 1 in. disk of thin white filter paper in the bottom; these disks are cut on a metal punching machine, many thicknesses at a time, but 0·7 in. squares could be used instead. The slides are put into the aluminium containers by slipping the container over the coated slide and unclipping the slide at the same time. A slight squeeze of the aluminium container makes this easier. As the container is brought upright the slide drops into the container and rests on the disk of filter paper at the bottom. The slide is not handled. The cap of the container is sealed with two 2 in. lengths of insulating tape (Rotunda P.V.C. adhesive tape $\frac{5}{8}$ in., Rotunda Ltd., Denton, Manchester). The slides are prepared under a half cylindrical dome of Perspex, hinged in two halves, attached to a baseboard 17 × 13 in., intended for the display of cheese (Code Number C.B.1, P. L. Display Co. Ltd., Southdown Works, Kingston Road, Raynes Park, London, S.W. 20).

The slides can be stored, preferably at 4° C., for a month or more: slides used after storage for a year at 4° C. have given the same results as freshly prepared slides. Difficulty with stored slides has arisen in the past from the growth of airborne mould spores contaminating the nutrient agar medium during preparation of the slides; because the container is opaque the contamination is not seen until the slide is removed for use. If the slides are carefully prepared only an occasional slide is contaminated, but the difficulty can be avoided by adding cycloheximide (Acti-dione, The Upjohn Company) to the nutrient agar medium at a concentration of 100 μg./ml. This was found to inhibit the growth of moulds on medium deliberately exposed to airborne contaminants, and such a concentration did not interfere with the growth of bacteria, including nine strains of coagulase negative staphylococci, isolated from pyogenic infections of the urinary tract, the number and size of the colonies being unchanged. We have found that the addition of cycloheximide to nutrient agar does not alter the relationship between dip-slide colony counts and pour plate viable counts (Guttmann & Naylor, 1967).

Method of use

The plastic tape sealing the container is peeled off and stuck down the side of the container for labelling. The slide is held in the fingers by the uncoated portion and dipped in the urine to immerse the agar medium. The slide is then drained by touching a corner to absorbent paper, which need not be sterile. The slide is replaced in the container, squeezing the container slightly if necessary, and the cap screwed on lightly; it is not resealed. The container should be left standing

upright for a few minutes so that any more urine which drains off the slide can be absorbed by the filter paper disk in the bottom of the container.

After delivery at the laboratory, the container is incubated overnight. The number of colonies on the slide can be counted, and the purity or otherwise of the growth assessed. Subcultures can be taken if necessary. The slide can be conveniently held in a pair of Spencer Wells forceps with short lengths of fine rubber tubing on the jaws. The slide should be discarded into disinfectant; if it is put back in the container, the lip and hence the rim of the cap may be contaminated by bacterial growth scraped off the slide.

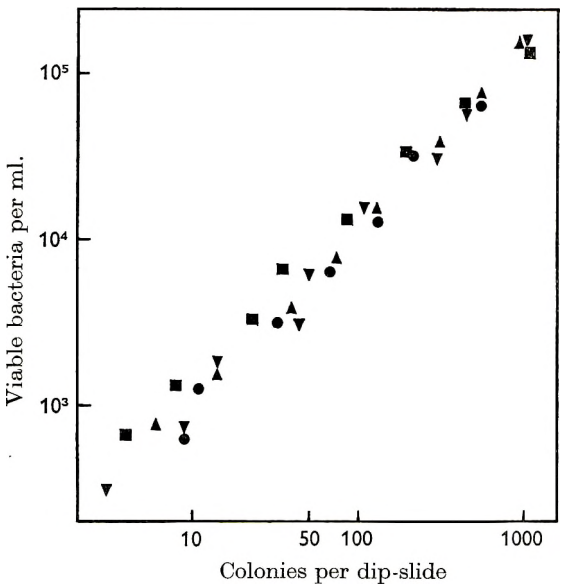


Fig. 1. Relationship between number of colonies on the dip-slide and the viable bacterial count of the fluid in which the slide has been dipped. ● *Escherichia coli*, ■ *Proteus mirabilis* (dip-slide counts and plate counts on MacConkey's agar). ▲ Faecal streptococcus, ▼ coagulase negative staphylococcus (dip-slide counts on nutrient agar and plate counts on blood agar).

RESULTS

Figure 1 shows the relationship between colony counts on dip-slides and viable bacterial counts of the fluid in which the slide is dipped. Overnight broth cultures of organisms from pyogenic infections of the urinary tract were diluted in quarter-strength Ringer's solution. Viable bacterial counts were determined by surface colony plate counts. All dip-slide and plate counts are the average of at least three determinations. The number of colonies on the dip-slide is directly proportional to the viable bacterial count of the fluid in which the slide has been dipped. Between 50 and 100 colonies on an area of 2 sq. in. on the dip-slide corresponds with a viable bacterial count of 10^4 per ml.

Colonies on the dip-slide are easy to count. Colony counts below 20 are unlikely to be reliable because of sampling variation; counts approaching and above 500 are increasingly difficult and inaccurate because of overcrowding of colonies;

above 1000 colonies the growth becomes semi-confluent. Therefore the dip-slide colony count is most reliable and convenient in the range from 20 to 500.

In general, contaminant bacteria in fresh urine are fewer than 10^4 per ml. whereas infecting organisms exceed 10^5 per ml. (Kass, 1956). Therefore the differentiation of viable bacterial counts below 10^4 or above 10^5 is of less importance in distinguishing between contamination and infection.

We have compared dip-slide colony counts with viable bacterial counts by the pour plate method on 385 samples of urine from patients attending a urinary infection clinic (Guttmann & Naylor, 1967). We found that fewer than 40 colonies on the dip-slide always corresponds with a pour plate viable count of less than 10^4 organisms per ml., and that viable counts greater than 10^5 organisms per ml. always correspond with more than 200 colonies on the dip-slide; heavily infected specimens containing more than 10^6 organisms per ml. give semi-confluent or confluent growths on the dip-slide. The dip-slide thus provides its maximum discrimination over the range of viable bacterial counts which is most helpful in the diagnosis of urinary tract infections.

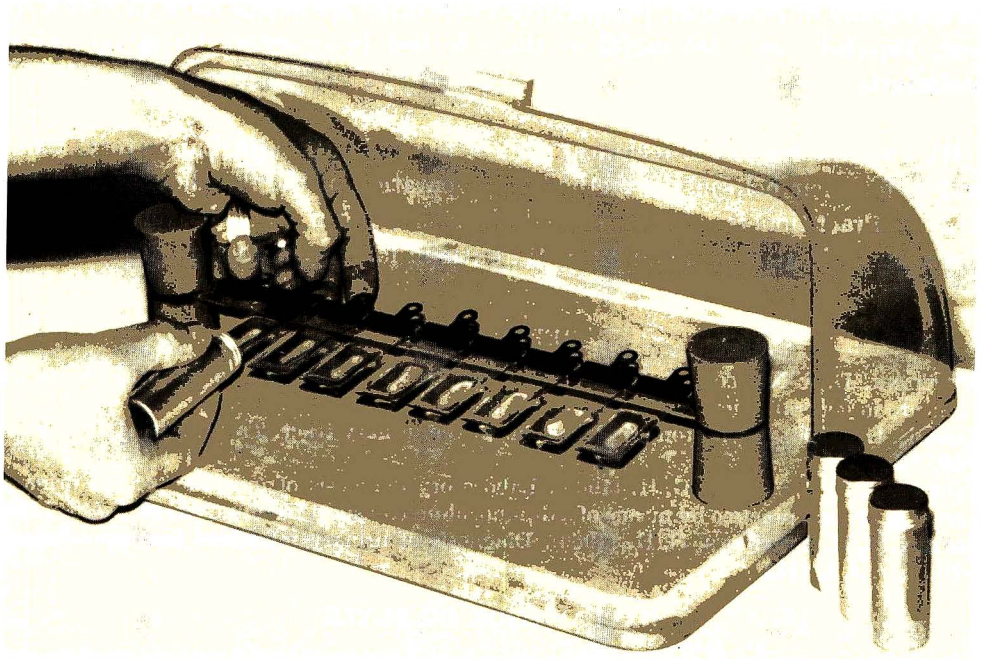
After a little experience, dip-slides can be used to estimate the numbers of viable bacteria in urine as significant, not significant or doubtful, without counting colonies. As the area of inoculated medium is 2 sq. in., features of colonies can be observed even when the urine contains many thousand organisms per ml.; consequently mixed growth can be detected as an additional indication of contamination.

Uses of dip-slides.

The dip-slide provides a measure of the bacterial concentration in freshly voided urine as supplementary information to that obtained by microscopy and conventional cultural methods including sensitivity tests. Dip-slides overcome difficulties in laboratory diagnosis caused by the proliferation of contaminating bacteria in urine after collection and consequently they are of value in the diagnosis of urinary tract infections in general practice where the laboratory is some distance away. Their main use is when urine specimens have to be sent to the laboratory by post, but they are also useful in evening surgeries. They are particularly helpful when there is any problem in diagnosis. They have been in use for a year without difficulty. They are unnecessary when urine specimens can be delivered quickly to the laboratory.

Dip-slides are also useful in screening for symptomless bacteriuria. When the majority of those to be examined are likely to be free from infection, culture of the urine on a dip-slide can be used as the sole test. In such circumstances, the ease of inoculating dip-slides and reading the results more than offsets the need to collect follow-up specimens for full examination from anyone giving a dip-slide result in the significant or doubtful range.

Dip-slides could also be useful in investigations in which it was desirable to follow the approximate bacterial concentration in urine at frequent intervals during the day and night. Dip-slides could be inoculated at intervals by nursing staff, and incubated and read in batches.



SUMMARY

The dip-slide, consisting of a glass microscope slide coated with nutrient medium and inoculated by dipping in freshly voided urine, provides a simple measure of the bacterial concentration in fresh urine. This is a useful supplement to the usual microscopy and culture of urine in the diagnosis of urinary tract infections in general practice, when specimens cannot be delivered to a laboratory within several hours of collection, particularly when urine specimens have to travel by post. Dip-slides are also useful as the sole test in screening for symptomless bacteriuria.

We are grateful to the technical staff of the Public Health Laboratory, Cambridge, for their help in the method of preparation of the dip-slides and to the Medical Practitioners who have used them and given us their comments. We thank Mr L. F. H. Beard and Mr S. W. Patman for the photographs.

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EXPLANATION OF PLATE

Rack for preparation of dip-slides. The slides have been coated with nutrient medium and an aluminium container is being slipped over a slide.

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Low-temperature preservation of *Leptospira*, preliminary communication

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INTRODUCTION

One of the main difficulties in running a service for the diagnosis of leptospirosis in man and animals is the maintenance of cultures of the various serotypes and strains for use as antigens. Present methods demand subculture in fluid medium at regular intervals of from 1 to 3 weeks, which is laborious and time consuming. Also, diminution in the viability of strains sometimes occurs and this may result in their loss. To avoid these difficulties, a reliable method of preparing a preserved stock of serotypes from which one could draw would be of value. Preservation of leptospires by freeze-drying has not proved satisfactory (Wolff, 1960), although Annear (1956, 1958) was able to recover leptospires in culture 2 years after drying them in high vacuum on sterile frozen-dried plugs of starch-peptone. These 'desiccates' stored in ampoules at 4° C. were more successfully preserved than those kept at room temperature. Maintenance of leptospires in tubes of semi-solid medium as recommended by Kirschner (1958) is more reliable in our experience but demands considerable storage space. So also does their maintenance in long narrow-bore tubes, such as tissue culture tubes, which minimize evaporation (using liquid medium to which 2 to 3 drops of fresh guinea-pig blood are added), a method recommended by L. H. Turner (WHO/FAO Leptospirosis Reference Laboratory, London). Weinman & McAllister (1947) described a method of preserving pathogenic protozoa by freezing suspensions of them in amounts up to 3 ml. in sealed Pyrex glass tubes, 100 × 13 mm. The tubes were cooled to −15° C. and then stored at '−70° C.' They included in their study a brief description of this treatment applied to several virulent strains of *Leptospira icterohaemorrhagiae* and to the saprophytic *Leptospira biflexa*; they obtained satisfactory growth in cultures from 9 out of 18 preparations after maintenance in a frozen state for periods ranging from 12 to 901 days. Wolff (1960) reported favourably on a similar method whereby a culture of leptospires in fluid medium was quickly frozen in a thin layer against the inner surface of a test tube (shell frozen) and then stored at low temperatures. He found that the organisms did not survive at −32° C., whereas at about −70° C., 39 of 48 strains were successfully subcultured after 1–7 years' storage. The virulence of the organisms was also maintained. Tarasevich, Bulk & Mudrova (1963) preserved four strains of pathogenic leptospires by a method similar to that of Weinman & McAllister (1947).

Their cultures grown in distilled water with 5 % rabbit serum were stored in 1 ml. amounts at −78° and −30° C. for periods ranging from 24 hr. to 6 months; the actual times of storage at the two specified temperatures are not made clear.

Polge & Soltys (1957) modified the method of Weinman & McAllister (1947) for the preservation of trypanosomes by adding glycerol to the suspension as a freezing protectant and by using a slow rate of cooling. By these means they obtained good survival rates for 8 months. The method has since been widely applied to the preservation of protozoal material. Detailed descriptions of apparatus and methods are available (Cunningham, Lumsden & Webber, 1963; Lumsden, Robertson & McNeillage, 1966). These workers developed methods that are very simple to operate and economical of space, as the suspensions of organisms are stored in glass capillary tubes.

Material preserved at low temperatures differs fundamentally from 'strain' material maintained in continuous reproduction by serial passage in that selection of the reproducing population is avoided and stabilization of biological characters may therefore be expected. This matter is discussed by Lumsden & Hardy (1965), who propose the term 'stabilate' for material preserved in this way. They define the term stabilate as 'a population of an organism preserved in a viable condition on a unique occasion'. The concept and methods had obvious application to the preservation of *Leptospira* and the present paper describes preliminary studies to this end.

MATERIALS

Twenty-seven different strains representing 11 serotypes have been studied. Details of these are given below. The nomenclature follows the World Health Organization's (1965) classification as far as possible. New isolates or strains of unknown origin are indicated by inverted commas.

Serotype	Strains
icterohaemorrhagiae	Wijnberg; Kantorowicz; 'O'Connor'; 'Downes'; 'Kirschner'
canicola	Hond, Utrecht IV; 'Welsh'; 'Cochrane'; 'Barnes'
ballum	Castellón 3; S 102
autumnalis	Akiyami A
bratislava	'H 10'; 'H 30'; 'H 41'; 'H 43'; 'H 102 A'
pomona	Pomona
grippotyphosa	Andaman CH 31
hardjo	Hardjoprajitno
sejroe	M 84; Mallersdorf II
saxkoebing	Mus 24
bataviae	van Tienen; 'Kearslie'; 'Young'; 'Kuching'; the last three were virulent strains of Sarawak origin

Some of these strains were provided by Prof. J. Wolff, Institute for Tropical Hygiene and Geographical Pathology, Amsterdam, and some by Dr L. H. Turner, London School of Hygiene and Tropical Medicine.

Strain 'Downes' was isolated in blood culture from a case of Weil's disease in a farm worker, strains 'Welsh' and 'Cochrane' from cases of canicola fever in pig farm workers and strain 'Barnes' from the urine of a pig on a farm associated

with another human case of canicola fever. These four strains were isolated in the Edinburgh area. Strains H10, H30, H41, H43 and H102A were isolated from the kidneys of hedgehogs, the first three caught near Edinburgh and the others near Compton, England (sent by Dr A. McDiarmid, Agricultural Research Council, Institute for Research in Animal Diseases, Compton, Berkshire). Strain designated 'Kirschner' was supplied by Dr Kirschner, Otago University, New Zealand.

METHODS

The leptospires were grown in small screw-capped bijou bottles (A. R. Horwell, Kilburn, London) containing 3 ml. modified Stuart's medium (Bryan, 1957) until they reached their maximum density, usually in 5–7 days. Before preservation, each culture was tested for freedom from contaminants by plating on blood agar. Since no antibiotics were used it was essential to apply strictly aseptic technique throughout the procedure.

The apparatus used for preservation was essentially the same as that described and illustrated by Cunningham *et al.* (1963) and Lumsden *et al.* (1966). The capillary tubes (lymph tubes, 4 in. long by 1 mm. outside diameter) were supplied by Messrs Plowden and Thompson, Stourbridge, England. The storage unit was made by Messrs E. K. Bowman, London, England; it can accommodate up to 12,000 tubes in 100 individually numbered compartments. This makes the locating of required material a simple procedure. The unit is filled with methanol and stored in a dry ice cabinet (Lumsden & Webb, 1961).

Glycerol was added to the culture to be preserved to give a final concentration of about 7·5 % by volume. The mixture was transferred to the capillary tubes held in a rack (Cunningham *et al.* 1963) by means of a Pasteur pipette and teat. The suspension flows into the tubes readily by capillary action and when a tube is about half filled the pipette is withdrawn and transferred to the next one and so on. When all the tubes have been so treated, the rack is tilted backwards and forwards so that the suspension comes to occupy the centre of each tube; the ends of the tubes are then sealed in a micro-burner. Care should be taken to ensure that the tubes are completely sealed. The tubes are taken from the rack and transferred to a cork-stoppered tube (120 mm. × 15 mm.) containing absolute methanol, which is then placed in an insulating jacket of Onazote (Expanded Rubber and Plastics, Ltd., Croydon, England) of walls 25 mm. thick and deposited in the dry ice cabinet. The rate of cooling of the material under these conditions has been defined by Lumsden *et al.* (1966): $-60°$ C., is reached in about 50 min. After 18–24 hr in the dry ice cabinet, when the temperature of the material will be approximately $-79°$C., the test tube is transferred quickly to a bath of methanol at $-79°$ C. for manipulation of the capillaries contained in it into the containers for permanent storage.

When a particular strain is required, one capillary tube is withdrawn from the appropriate compartment in the storage unit and wiped free of alcohol with a sterile pad of cotton-wool. Each end of the tube is carefully marked with a glass cutter and broken off and passed quickly through a gas flame. The contained

suspension, now thawed, is blown out into bottles containing 3 ml. Stuart's medium. This is done aseptically by means of a perforated rubber teat attached to one end of a 5 cm. length of glass tubing plugged with cotton-wool (Fig. 1). The other end of the tube is fitted with a soft rubber stopper of the kind used for vaccine

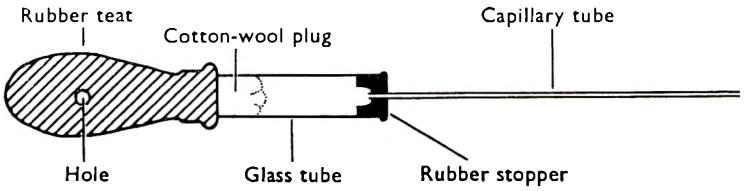

Fig. 1. Device used for transferring contents of capillary tube aseptically to culture medium.

Table 1. *Recovery rate of* 21 *strains of* Leptospira *preserved by freezing during the period* 7 *February–*9 *June* 1964

		No. of months frozen									
Serotype	Strain	4	5	6	7	8	12	18	24	27	Total
canicola	Welsh	1/1	—	2/2	3/3	—	1/2	—	—	0/4	7/12
ballum	S 102	1/1	—	2/2	1/3	—	—	—	—	—	4/6
canicola	Hond Utrecht IV	—	—	3/3	2/2	2/3	—	—	—	0/4	7/12
saxkoebing	Mus 24	—	2/3	1/3	0/1	—	—	—	—	—	3/7
ictero-haemorrhagiae	Wijnberg	—	—	1/3	4/4	1/1	—	2/2	—	4/4	12/14
ictero-haemorrhagiae	Kantorowicz	—	3/3	—	—	—	—	—	0/4	—	3/7
pomona	Pomona	—	—	2/2	—	—	—	—	0/4	—	2/6
bratislava	H 10	—	—	2/2	—	—	—	—	—	0/4	2/6
sejroe	M 84	—	2/2	1/1	2/3	—	—	—	—	—	5/6
sejroe	Mallersdorf II	—	2/2	—	—	—	—	—	0/4	—	2/6
grippotyphosa	Andaman CH 31	—	2/2	3/3	1/1	—	—	—	—	4/4	10/10
ictero-haemorrhagiae	Downes	—	3/3	2/2	1/1	—	2/2	—	—	—	8/8
hardjo	Hardjo-prajitno	—	—	2/2	—	—	—	2/2	—	—	4/4
bratislava	H 102 A	—	2/2	—	—	—	—	0/2	—	—	2/4
ballum	Castellón 3	—	2/2	3/3	—	—	—	—	—	—	5/5
autumnalis	Akiyami A	—	2/2	—	—	—	—	—	—	—	2/2
ictero-haemorrhagiae	Kirschner	—	—	2/2	—	—	—	2/2	—	—	4/4
bataviae	van Tienen	—	2/2	—	—	—	0/2	—	—	—	2/4
canicola	Cochrane	1/2	—	—	—	—	—	—	—	—	1/2
canicola	Cochrane	1/2	—	—	—	—	—	—	—	—	1/2
bratislava	H 30	2/2	—	—	—	—	—	—	—	—	2/2

Percentage number of cultures satisfactory, 68.

or dental anaesthetic tubes. A small hole is pierced in the rubber stopper so that the end of the capillary tube can be inserted into it (a number of these tubes with

stopper are sterilized in a container ready for use). By applying finger pressure to close the hole in the teat and to compress the teat, the suspension in the lymph capillary can be blown out of the other end into the medium.

RESULTS

Between 7 February and 9 June 1964, capillaries of 21 different strains of *Leptospira* were frozen and then stored for periods ranging from 4 months to $2\frac{1}{4}$ years (Table 1). Every one of the strains was recovered after freezing, although not all of them survived for the same length of time.

Table 2. *Recovery rate after preservation by freezing of eight cultures aged* 3–8 *weeks*

Serotype	Strain	Age (weeks)	Period of storage months	Recovery rate: Living organisms seen in culture	Recovery rate: Satisfactory multiplication in culture
icterohaemorrhagiae	Kearlsey	4	2	2/2	2/2
			19	2/2	0/2
		8	5	2/2	0/2
			6	—	0/2
			7	—	0/2
			19	0/2	0/2
bataviae	Young	3	2	2/2	2/2
			19	2/2	0/2
		3	5	2/2	0/2
			6	—	0/2
			7	—	0/2
			19	1/1	0/2
bataviae	O'Connor	4	2	2/2	2/2
			19	1/2	0/2
		4	5	2/2	0/2
			6	—	0/2
			7	—	0/2
			19	2/2	0/2
bataviae	Kuching	5	2	2/2	2/2
			19	1/2	0/2
		5	5	1/2	0/2
			6	—	0/2
			7	—	0/2
			19	1/1	0/2

With one exception, growth resulted from the addition of the contents of one capillary tube to the culture medium; in one case two tubes were necessary. Not every tube has resulted in growth, some of the tubes were improperly sealed and the content of others was contaminated, probably by faulty manipulation at the time of culturing the thawed material. A total of 129 attempts to culture the preserved leptospires were made of which 88 (68%) were successful. Eight of 20 cultures prepared from leptospires preserved for $2\frac{1}{4}$ years resulted in satis-

factory growth of density equivalent to the original culture after 10 days' incubation; seven others were seen to contain a few motile leptospires but these failed to multiply even in subculture.

During January 1965, eight additional cultures of four strains of leptospira (1 *icterohaemorrhagiae* and 3 *bataviae*) were preserved by freezing. The ages of these cultures ranged from 3 to 8 weeks. They were tested for survival and ability to multiply at periods of 2–19 months after freezing. The results are given in Table 2.

Cultures of leptospires that were 3, 4 and 5 weeks old when frozen were satisfactorily cultured 2 months later. None of them, however, could be cultured after periods of 5–19 months' storage although active organisms were seen in 17 out of 22 of the cultures.

In all, 177 cultures have been attempted. Ninety-six (54 %) were satisfactory and 81 (46 %) failed to produce growth.

DISCUSSION

The results presented indicate that the long-term viable preservation of *Leptospira* materials is possible by storing suspensions in glass capillary tubes at − 79° C. In most cases the organisms survived and could be re-established in culture for periods of up to over 2 years. The possible practical applications of such a method of preservation are obvious. It should be possible to lay down standard materials available for reference over long periods—the concept of stabilates as discussed by Lumsden & Hardy (1965). For this, further studies are required: quantitative to define the proportions of the organisms which ultimately survive the processes of preservation, storage and retrieval from preservation: and qualitative to establish the concordance of the characteristics of the organisms before and after treatment.

The factors that determine survival require fuller study. Even in the cases in which attempts to recover leptospires in culture were unsuccessful, actively motile leptospires could often be observed by dark-ground microscopy. Some may even have reproduced to some extent before dying out as motile leptospires were observed in subculture. The factors which seem likely to influence success are, in our consideration: the age of the culture when preserved and the quality of the culture medium used.

The use of a chemically-defined medium and the possibility of additional growth factors to stimulate multiplication of the organisms that have been devitalized by the freezing process would no doubt improve the reliability of the procedure.

SUMMARY

1. A simple method for the long-term viable-preservation of samples of *Leptospira* suspensions is described. Quantities of about 25 μl., with the addition of 7·5 % glycerol, are introduced into capillary tubes, cooled slowly to − 79° C. and stored at that temperature.

2. More than half the attempts were successful in re-establishing growth in culture after storage at − 79° C. for periods up to 27 months.

3. The possibility that the method could be used for establishing 'banks' of standard *Leptospira* material for reference is discussed.

Mrs Jean Smith contributed greatly to the work by her painstaking carrying out of the preparation and examination of cultures.

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A prospective study of staphylococcal infection and its prevention among infants and mothers after childbirth in hospital and at home

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In a previous investigation it was shown that infants who became heavily colonized by *Staphylococcus aureus* soon after birth subsequently developed septic infections more often than other infants (Gillespie, Simpson & Tozer, 1958). Applications of 'Ster-zac' hexachlorophane powder to umbilicus, trunk and flexures greatly reduced both staphylococcal colonization and infection in infants and mothers (Simpson, Tozer & Gillespie, 1960; Corner, Crowther & Eades, 1960). Hexachlorophane powder has been in routine use since 1959 in the Bristol Maternity Hospital and the obstetric wards of Southmead Hospital. The incidence of staphylococcal disease has remained low and the treatment has caused no ill effects in patients or staff.

The purpose of this paper is to report a more extensive investigation of colonization and infection by *Staph. aureus* during the first 6 months after childbirth in hospital and at home. Infants, mothers and members of households were studied. The prophylactic value of hexachlorophane was confirmed and the effect of disinfecting infants' noses with 'Naseptin' cream was investigated. Results of a previous investigation, hitherto unpublished, are also reported.

MATERIALS AND METHODS

The principal survey included all infants born in the Bristol Maternity Hospital (B.M.H.) and in one ward of Southmead Hospital (S.H.) between 1 November 1960 and 30 April 1962, with the exception of premature, illegitimate and multiple births. Infants who left hospital before the 8th or after the 12th day of life were also omitted from the survey, but any who stayed longer in hospital because of staphylococcal disease were included. Infants who were born at home on even dates throughout the same period constituted the domiciliary group.

A total of 1707 infants and mothers entered the survey, and 165 left it before completing 6 months—93 because they moved from Bristol, 51 because of non-cooperation, 18 because information was inadequate and 3 infants who died. No death was caused by staphylococci. No mother died. The 1542 who remained in the survey for 6 months consisted of 712 who were born at home, 267 in Southmead Hospital, 351 in Floor 2 and 212 in Floor 3 of Bristol Maternity Hospital.

Records were kept of staphylococcal disease in infants, mothers and other members of households. Lesions were classified as pyoderma (pustules, boils, styes and paronychiae), maternal mastitis, staphylococcal otitis, and staphylococcal conjunctivitis. Provision was made to record other lesions such as pemphigus neonatorum, deep abscess, osteomyelitis, and staphylococcal pneumonia, but none was observed. Purulent lesions of skin or breast were counted as staphylococcal even when cultures were not performed. Mastitis which resolved without suppuration during antibiotic treatment was counted as staphylococcal.

Conjunctivitis ('sticky eye') was regarded as staphylococcal only when culture yielded a profuse growth of *Staph. aureus*.

Lesions which started in the first two weeks of life were seen by doctors in hospital and by doctors or midwives at home and nearly all were swabbed. Health visitors called on the families in the sixth week and sixth month. They saw all mothers and babies, inquired about illnesses in the households and inspected and swabbed all lesions. In addition, mothers were asked to notify immediately any lesion, however trivial, using pre-addressed stamped postcards, and to see their family doctors. The doctors reported lesions which came to their notice and swabbed any that were discharging.

Saline-moistened nose swabs were taken from mothers on admission to the Bristol Maternity Hospital and from mothers and infants of all groups between the 8th and 12th post-partum days ('2nd week swabs'). The swabs were repeated 6 weeks and 6 months after delivery. Infants who were treated with nasal cream were swabbed 24 hr. after its last application. The bacteriological methods were described by Simpson *et al.* (1960). Most staphylococci were phage-typed (Anderson & Williams, 1956).

The nature of illnesses requiring admission to hospital was determined and the causes of all deaths ascertained by *post mortem*. Factors which might have influenced susceptibility to infection were recorded; these were sex, season of birth, maternal age, number and ages of others in household, parents' occupations, district, Registrar General's social class, quality of mothercraft, breast or artificial feeding and the types of baby powders and creams used at home. Data were entered on special forms by midwives, health visitors and bacteriologists and after coding were transferred to punched cards for analysis.

Prophylactic disinfectants

In accordance with routine practice, the umbilicus, trunk, groin and perineum of every infant in hospital were liberally dusted with 'Ster-zac' powder about 6 hr. after birth and again every time their napkins were changed (Simpson *et al.* 1960). The powder contains hexachlorophane 0·33 %, in a pre-sterilized base. In addition, infants in Floor 2 of Bristol Maternity Hospital were treated once a day with 'Naseptin' nasal cream, which was smeared inside the anterior nares by means of sterile glass rods, followed by gentle massage of the nose. The cream contains neomycin 0·5 % and chlorhexidine 0·1 %. The effect of 'Naseptin' alone was not studied because it was not considered justified to stop the use of hexachlorophane.

Mothers were not advised to use any particular baby powder after leaving hospital, but some continued to use 'Ster-zac' for varying periods. 'Naseptin' was not used outside hospital.

In the domiciliary group, 'Ster-zac' was applied to the umbilicus during the first two weeks of life.

RESULTS

Analysis of factors that might have influenced susceptibility to infection revealed the following differences between the groups. In the domiciliary group, mothers were on average older than those in hospital and had larger households and higher proportions were in social classes 4 and 5. Mothers in Southmead Hospital were on average older, their parity was greater and their social class lower than in Bristol Maternity Hospital. There were more primagravidae and a smaller average size of household in Floor 3 than in Floor 2 of Bristol Maternity Hospital. The proportions of mothers who breast-fed their infants did not differ greatly (Table 1). None of these differences could be shown to influence staphylococcal colonization or infection.

Table 1. *Percentage of mothers who breast-fed their infants*

	Place of birth			
	Hospital			
	B.M.H.			
Duration of breast feeding	Floor 2	Floor 3	S.H.	At home
At least 2 weeks	84	88	77	80
At least 6 weeks	48	46	30	38
At least 6 months	18	14	10	10

Staphylococcal infection in infants

The incidence of staphylococcal disease was low in all groups (Table 2). No infection was severe nor required admission to hospital.

Previous surveys in both hospitals showed that the incidence of pyoderma during the first 2 weeks of life fell promptly to about one-fifth of its former value when hexachlorophane was introduced in 1959. Comparison with infants of the present series during their first 2 weeks showed that infection rates had remained low and confirmed the value of hexachlorophane (Table 3). Comparison of the B.M.H. groups in the present series (Table 2) showed that the use of 'Naseptin' in addition to hexachlorophane was accompanied by a further significant reduction of infection. A comparison of the total staphylococcal infections in the two floors gave a significant difference ($P = 0{\cdot}0196$ by Fisher's Exact Probability Test). The infection rate in Floor 2, where both hexachlorophane and 'Naseptin' were used, was almost identical with the domiciliary rate.

Table 2. *Staphylococcal disease in infants during first 6 months of life* (1960–62)

Place of birth	Disinfectant treatment	No. of infants observed for 6 months (M = male, F = female)	Infants with staphylococcal lesion		
			Pyoderma	Conjunctivitis and otitis	Total infants infected
At home	Hexachlorophane to umbilicus	M 377	4	1	5
		F 335	5	4	7
		Total 712	9 (1·3 %)	5 (0·7 %)	12* (1·7 %)
B.M.H. 2	Hexachlorophane to umbilicus, groin and perineum. 'Naseptin' to nose	M 117	2	0	2
		F 174	2	3	5
		Total 351	4 (1·1 %)	3 (0·9 %)	7 (2·0 %)
B.M.H. 3	Hexachlorophane to umbilicus, groin and perineum	M 97	8	5	12
		F 115	0	0	0
		Total 212	8 (3·8 %)	5 (2·4 %)	12* (5·7 %)
S.H.	Hexachlorophane to umbilicus, groin and perineum	M 138	3	1	4
		F 129	3	1	4
		Total 267	6 (2·2 %)	2 (0·7 %)	8 (3·0 %)

* Some of these infants had pyoderma and conjunctivitis.

Sex distribution of infection

Elias-Jones, Gordon & Whittaker (1961) recorded a moderate but statistically significant excess of staphylococcal infection in male infants. Helms & Stenderup (1961) found no difference between boys and girls who were born in hospital, but slightly more boys than girls were infected after domiciliary delivery. Thompson *et al.* (1963) found a large excess of infection in males and suggested that it might be related to circumcision (Gezon *et al.* 1964). Plueckhahn & Banks (1964*a*) also found more infection in males, though few were circumcised. Williams (1964) found slightly more infections in boys, though the excess was not statistically significant.

In the present study, no sex difference was found except in B.M.H. Floor 3, where all pyoderma lesions were in boys (Table 2). The reason for the difference is not known. No infants were circumcised while in hospital.

Table 3. *Effect of hexachlorophane powder on the incidence of pyoderma in the first 2 weeks of life*

	Previous investigations* (1957–60)		Present investigation (1960–62)
Disinfectant treatment	None	Hexachlorophane	Hexachlorophane
	Incidence of pyoderma		
Hospital			
B.M.H. Floor 2	19/365 (5·2 %)	5/554 (0·9 %)	0/351 ('Naseptin' was also used)
B.M.H. Floor 3	34/464 (7·3 %)	—	5/212 (2·4 %)
S.H.	277/3721 (7·4 %)	54/3650 (1·5 %)	1/267 (0·4 %)

* See Simpson *et al.* (1960) and Corner *et al.* (1960).

Infection in mothers and households

The incidence of staphylococcal disease in mothers was low and similar in all groups (Table 4). This result differed markedly from that observed before hexachlorophane was introduced, when mastitis was much commoner after hospital than domiciliary delivery (Corner *et al.* 1960). All but one of the cases of mastitis were in mothers who breast-fed their infants for at least 1 week.

Infection usually came later in mothers than in infants, 84 % of maternal lesions beginning after the second post-partum week, compared with 44 % of infants.

The incidence of staphylococcal disease in other members of households did not differ significantly between the groups.

Phage patterns of the staphylococci

As previously observed (Gillespie *et al.* 1958), most hospital infections were by phage group I strains, usually penicillin-resistant. Few were resistant to other

Table 4. *Staphylococcal disease in mothers during the 6 months after delivery*

Where delivered	Disinfectant treatment of infants	No. of mothers observed for 6 months	Mothers with staphylococcal disease			
			Pyoderma	Mastitis	Otitis	Total
At home	Hexachlorophane to umbilicus	712	13 (1·8%)	7 (1·0%)	0	20 (2·8%)
B.M.H. 2	Hexachlorophane to umbilicus, groin and perineum. 'Naseptin' to nose	351	6 (1·7%)	2 (0·6%)	0	8 (2·3%)
B.M.H. 3	Hexachlorophane to umbilicus, groin and perineum	212	4 (1·9%)	3 (1·4%)	0	7 (3·3%)
S.H.	Hexachlorophane to umbilicus, groin and perineum	267	7 (2·6%)	1 (0·4%)	1 (0·4%)	8 (3·0%)
	Total hospital deliveries	830	17 (2·0%)	6 (0·7%)	1 (0·1%)	23 (2·8%)

Table 5. *Pyoderma in infants during the first 6 months of life in relation to nasal carriage of staphylococci in the second week*

Place of birth...	At home	B.M.H 2	B.M.H. 3	S.H.	All		
Nose swab (2nd week)	Incidence of pyoderma (number of infants infected/number in group)						
					During 1st 2 weeks	After 2nd week	Total
Staph. aureus absent	2/380	3/244	1/90	2/169	2/883	6/883	8/883 (0·9%)
Staph. aureus present	7/312	1/57	6/98	4/91	7/558	12/558	18/558 (3·2%)
Staph. aureus present (penicillin resistant)	2/113	1/37	5/58	4/52	4/260	8/260	12/260 (4·6%)
Staph. aureus present (penicillin sensitive)	5/199	0/20	1/40	0/39	3/298	4/298	6/298 (2·0%)
Not swabbed	0/20	0/50	1/24	0/7	0/101	1/101	1/101 (1·0%)
Total	9/712	4/351	8/212	6/267	9/1542	19/1542	27/1542 (1·8%)

The totals for the two periods do not always agree with the total number of infants infected. One infant with penicillin sensitive *Staph. aureus* in its swab had an attack of pyoderma in the first two weeks and another attack later.

antibiotics. There was no predominant epidemic strain. Several mothers were infected by strains which had colonized their infants.

Most domiciliary infections were caused by group II strains which usually were penicillin sensitive.

Effect of infants' staphylococcal carriage on infection among infants and mothers

As previously demonstrated, infants who quickly became staphylococcal carriers developed pyoderma more often than others. In the present series 3·2 % of infants who were nasal carriers in the 2nd week suffered from pyoderma, compared with 0·9 % of non-carriers (Table 5). The difference was highly significant: by Fisher's Exact Probability Test, $P = 0{\cdot}00145$. The incidence of sepsis was slightly but not significantly higher among carriers of penicillin-resistant than of sensitive strains, $P = 0{\cdot}067$.

The incidence of mastitis was 1·8 % in mothers of nasal carriers compared with 0·1 % in mothers whose infants were not carriers (Table 6). This difference was highly significant; by Fisher's Exact Probability Test, $P = 0{\cdot}00052$. In those who were swabbed, there was only one case of mastitis among mothers of infants who were not carriers when 2 weeks old.

Sepsis other than mastitis was only slightly and not significantly commoner in mothers of nasal carriers (Table 6).

Comparison of infection rates in the B.M.H. groups (Table 2) showed that the use of 'Naseptin' in addition to hexachlorophane gave increased protection against sepsis in infants. Comparison with earlier reports suggested that hexachlorophane played the greater part (Table 3). This was consistent with the fact that the umbilicus and flexures are generally colonized before the nose, at an age when the skin is very susceptible to infection (Gillespie *et al.* 1958). Jennison & Komrower (1961) showed that 'Naseptin' by itself reduced nasal colonization and slightly reduced skin infection.

There were slightly fewer infections in mothers of infants who were treated with 'Naseptin' than in other mothers (Table 4), but the difference was not significant. However, the relationship between maternal mastitis and infants' nasal carriage, described above, showed that the treatment of infants must have protected some mothers from breast abscess.

Influence of disinfectant treatment of infants on staphylococcal nasal carriage by infants and mothers

After the first week or two of life the infant's nose is perhaps the principal source from which staphylococci are transmitted from the maternity hospital to the community outside. It was hoped that nasal disinfectant treatment would reduce this source of dissemination and to some extent it did so (Table 7). At the age of 2 weeks infants in B.M.H. Floor 2, where 'Naseptin' was used, had a nasal carrier rate of 19 % compared with 51 % in Floor 3. This difference is highly significant ($\chi^2 = 64{\cdot}637$; $P < 0{\cdot}001$). By the 6th week, the rates were 43 % and 53 % respectively, and the difference was still significant ($\chi^2 = 5{\cdot}294$; $P < 0{\cdot}05$). There was no significant difference between the rates at 6 months, which by then

Table 6. *Staphylococcal disease in mothers in relation to nasal carriage of Staph. aureus by their infants in the second week of life*

Place of delivery	At home	In hospital B.M.H. 2	In hospital B.M.H. 3	In hospital S.H.	All		
	Incidence of disease						
	P* M*	P M	P M	P† M	Pyoderma†	Mastitis	Total
Infant's nose swab							
Staph. aureus absent	7 + 1 / 380	4 + 0 / 244	2 + 0 / 90	2 + 0 / 169	15 (1·7%)	1 (0·1%)	16 (1·8%) / 883
Staph. aureus present	6 + 6 / 312	1 + 0 / 57	2 + 3 / 98	4 + 1 / 91	13 (2·3%)	10 (1·8%)	23 (4·1%) / 558
Staph. aureus present (penicillin resistant)	1 + 2 / 113	0 + 0 / 37	1 + 1 / 58	2 + 0 / 52	4 (1·5%)	3 (1·2%)	7 (2·7%) / 260
Staph. aureus present (penicillin sensitive)	5 + 4 / 199	1 + 0 / 20	1 + 2 / 40	2 + 1 / 39	9 (3·2%)	7 (2·4%)	16 (5·4%) / 298
Not swabbed	0 + 0 / 20	1 + 2 / 50	0 + 0 / 24	1 + 0 / 7	2 (2·0%)	2 (2·0%)	4 (4·0%) / 101
Total	13 + 7 / 712	6 + 2 / 351	4 + 3 / 212	7 + 1 / 267	30 (1·9%)	13 (0·8%)	43 (2·8%) / 1542

* *P* = pyoderma. M = mastitis

† Including 1 case of otitis in a mother whose infant carried penicillin resistant *Staph. aureus*.

Table 7. *Staphylococcal nasal carriage by infants and mothers* 2 *weeks,* 6 *weeks and* 6 *months after delivery* (1960–62)

Where delivered	Infants			Mothers		
	2 weeks	6 weeks	6 months	2 weeks	6 weeks	6 months
At home	327/727 (45%)	257/706 (36%)	133/701/(19%)	264/710 (37%)	279/703 (40%)	260/692 (38%)
Hospital						
B.M.H. 2	63/334 (19%)	154/359 (43%)	77/341 (23%)	113/331 (34%)	164/358 (46%)	137/341 (40%)
B.M.H. 3	113/220 (51%)	115/218 (53%)	50/205 (24%)	75/218 (34%)	100/221 (45%)	84/204 (41%)
S.H.	101/283 (36%)	123/273 (45%)	56/256 (22%)	87/279 (31%)	128/271 (47%)	105/253 (42%)

The disinfectant treatment of infants is shown in Table 2.

Table 8. *Staphylococcal nasal carriage by infants and mothers in relation to disinfectant treatment of infants (investigations of* 1959)

Treatment	Infants. Proportions of swabs positive		Mothers. Proportions of swabs positive		
	2 weeks after birth	6 weeks after birth	On admission to hospital	2 weeks after delivery	6 weeks after delivery
Group 1. Non-disinfectant powder	42/47 (89 %)	82/107 (77 %)	31/91 (34 %)	54/109 (49 %)	99/132 (75 %)
Group 2. Hexachlorophane powder	28/52 (54 %)	36/67 (54 %)	30/90 (33 %)	33/82 (40 %)	33/69 (48 %)
Group 3. Hexachlorophane powder and 'Naseptin'	9/96 (9 %)	55/168 (33 %)	73/210 (35 %)	51/152 (34 %)	32/198 (41 %)

Tests of significance for the differences between carrier rates

	Infants	Mothers
Between groups (1) and (2)	At 2 weeks $\chi^2 = 15{\cdot}034$, $P < 0{\cdot}001$	At 2 weeks $\chi^2 = 1{\cdot}631$, Not significant
	At 6 weeks $\chi^2 = 9{\cdot}903$, $P < 0{\cdot}01$	At 6 weeks $\chi^2 = 14{\cdot}842$, $P < 0{\cdot}001$
Between groups (2) and (3)	At 2 weeks $\chi^2 = 35{\cdot}577$, $P < 0{\cdot}001$	At 2 weeks $\chi^2 = 1{\cdot}036$, Not significant
	At 6 weeks $\chi^2 = 8{\cdot}896$, $P < 0{\cdot}01$	At 6 weeks $\chi^2 = 0{\cdot}858$, Not significant
Between groups (1) and (3)		At 2 weeks $\chi^2 = 6{\cdot}749$, $P < 0{\cdot}01$
		At 6 weeks $\chi^2 = 36{\cdot}075$, $P < 0{\cdot}001$

had fallen to less than 30% in all groups, in accordance with the findings of Ludlam (1953). The nasal carriage rates in all four groups of mothers were almost identical at the three times of swabbing (Table 7).

The previous investigation

The actions of hexachlorophane and 'Naseptin' on staphylococcal nasal carriage were shown more clearly in an earlier investigation. Three groups of infants and their mothers were studied in the Bristol Maternity Hospital in 1959. In one group, the control, infants were treated for 2 weeks with a non-disinfectant dusting powder ('Ster-zac' base) at every napkin change; in another group they were treated with 'Ster-zac' hexachlorophane powder and in a third with 'Naseptin' nasal cream as well as 'Ster-zac'. The cream was applied twice a day, i.e. more

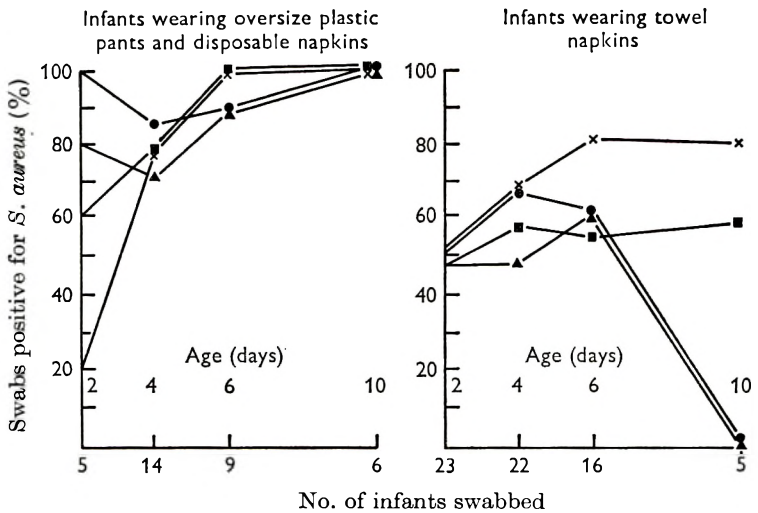

Fig. 1. Influence of clothing on staphylococcal colonization of newborn infants. ×——×, Nose; ●——●, umbilicus; ■——■, perineum; ▲——▲, groin.

often than in the subsequent investigation. Four weeks after stopping treatment the hexachlorophane-treated infants still had a significantly lower nasal carriage rate than the control group. The group treated with hexachlorophane and 'Naseptin' had an even lower rate. The mothers of both groups of treated infants had significantly lower carriage rates than those of the control group (Table 8).

Skin moisture and staphylococcal carriage

Staphylococci on newborn infants' skin are usually most numerous in the umbilical area, groin, perineum and other flexures. Before hexachlorophane treatment was instituted, swabs from these areas often gave profuse growths of staphylococci, whereas those from the back of the neck and thorax gave only scanty growth or were negative. The moist skin of flexures appeared to encourage staphylococcal multiplication. Support for this belief was inadvertently obtained during a short period in 1959 when infants in a nursery where hexachlorophane

was not used were dressed in plastic waterproof pants worn outside their napkins. The pants were too large and covered most of the thorax as well as abdomen and thighs. During this period staphylococcal colonization increased (Fig. 1). Profuse growths were obtained from the front and back of the thorax, areas which normally yielded few staphylococci. Subsequently it was found that smaller pants could be worn without encouraging the growth of staphylococci, at any rate when the infants were treated with hexachlorophane powder.

DISCUSSION

The newborn infant's umbilicus, skin flexures and nose are remarkably susceptible to colonization by *Staph. aureus*. Susceptibility may be further increased by unsuitable garments which cause excessive moisture of the skin. The staphylococci, derived originally from adults, are often transmitted from baby to baby in hospital. The fact that disinfection of nurses' hands reduced cross-infection among infants showed that handling is an important mode of transmission (Gillespie *et al.* 1958).

The more rapid and profuse the colonization of an infant the more liable are he or his mother to clinical infection. Most sites of staphylococcal colonization of infants are superficial and accessible to topical disinfection. Repeated application of hexachlorophane to skin and umbilicus reduces colonization and consequently infection. The remarkable protection afforded by hexachlorophane described by Farquharson, Penny, Edwards & Barr (1952), has been amply confirmed (Simpson *et al.* 1960; Payne, Wood, Karakawa & Gluck, 1965). It contrasts strikingly with the prophylactic failure of systemic antibiotics described by Forfar *et al.* (1966).

Treatment of infants with hexachlorophane reduces the incidence of breast abscess in mothers (Corner *et al.* 1960; Plueckhahn & Banks, 1964*b*).

Most authors have described the use of hexachlorophane in baths. Dusting with a pre-sterilized powder containing hexachlorophane may be used instead by those who prefer to avoid frequent bathing of infants before the umbilical cord stump separates. Our experience and that of Hughes (1961) showed that the powder is convenient and effective. It must be applied, at every napkin change (i.e. 6–8 times a day), to the umbilical area, perineum and flexures.

Hexachlorophane treatment of skin and umbilicus reduced nasal carriage, probably because the nose is often colonized by staphylococci from the umbilicus (Gillespie *et al.* 1958). Nasal carriage and sepsis were further reduced when infants were treated with 'Naseptin' nasal cream in addition to hexachlorophane. The incidence of maternal breast abscesses, already very low in all groups, was not significantly less in mothers of infants who were treated with 'Naseptin'. But since breast abscesses were almost entirely confined to mothers whose infants were nasal carriers by the second week, the risk of this complication must have been reduced by 'Naseptin'.

Staphylococcal sepsis in many centres, including Bristol, in the past has been commoner after hospital than after domiciliary delivery. Such differences can be abolished by an efficient antiseptic regime.

The diminution of nasal carriage in infants which resulted from their treatment with disinfectants in hospital persisted after they went home. Colonization of mothers' noses was also diminished and the spread of hospital staphylococci into the general population presumably was reduced.

Of the two prophylactic agents studied in these trials, hexachlorophane was the more beneficial and was easier to use. 'Naseptin' was difficult to apply correctly and required frequent supervision of the nursery staff. It is therefore doubtful whether the routine use of 'Naseptin' is worth while. It might be valuable as an occasional supplement to hexachlorophane, for example, to control cross-infection by an unusually virulent or transmissible staphylococcus, except when the strain is resistant to neomycin.

SUMMARY

Staphylococcal colonization and infection were studied prospectively in infants, mothers and households after childbirth at home and in hospital. Infants were treated prophylactically with frequent applications of 'Ster-zac' hexachlorophane dusting powder. Some were treated in addition with 'Naseptin' nasal disinfectant cream.

The incidence of staphylococcal sepsis in infants was much less than before the adoption of hexachlorophane prophylaxis. The sepsis rate was further reduced when 'Naseptin' was used in addition to hexachlorophane. Of the two prophylactic agents, hexachlorophane was the more convenient and probably the more effective. 'Naseptin' was difficult to employ correctly and unsuitable for routine use.

The treatment of infants with disinfectants reduced nasal carriage markedly in infants and to a smaller extent in mothers. Both agents contributed to the reductions which persisted for some weeks after treatment ceased.

Breast abscesses were almost entirely confined to mothers of infants who became nasal carriers by the second week of life.

Staphylococcal colonization of infant's skin was greater when they wore impervious garments, probably because skin moisture increased.

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The incidence of *Herpesvirus hominis* antibody in the population

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INTRODUCTION

For a number of years the results of Buddingh, Schrum, Lanier & Guidry (1953) in New Orleans, U.S.A. have been quoted in the textbooks as showing that 90 % of the population over the age of 15 have neutralizing antibody to *Herpesvirus hominis*. The most complete British survey, reported by Holzel, Feldman, Tobin & Harper (1953), using a complement-fixation technique, showed a similar high incidence of antibody (86 %) in the same age groups.

These findings have been supported by many other surveys, although it has become apparent that the socio-economic background of the individuals in such a survey has an important influence on the detected incidence of antibody (Zinsser & Tang, 1929; Andrewes & Carmichael, 1930; Weyer, 1932; Burnet & Williams, 1939; Anderson & Hamilton, 1949; Hayward, 1949; Buddingh *et al.* 1953; Holzel *et al.* 1953; Coetzee, 1955; Dascomb, Adair & Rogers, 1955; Halonen, 1955; MacCallum, 1959; Stern, Elek, Millar & Anderson, 1959; Yoshino *et al.* 1962; Taglieri & Tresca, 1964; Kibrick & Gooding, 1965; Rodrigues & Carvalho, 1965; Becker, 1966).

In view of the suggestion of Yoshino *et al.* (1962) that a change in age distribution of antibody had occurred in Japan and as a period of 12 years had elapsed since the Holzel *et al.* (1953) report, it was thought worth while to determine the present incidence of both neutralizing and complement-fixing antibody in two populations in this country.

MATERIALS AND METHODS

Virus

A wild strain of *Herpesvirus hominis* producing rounded, ballooning degeneration of the tissue cells was used for the preparation of both the neutralizing and complement-fixing antigen in Edinburgh. A similar wild strain was used in the neutralization tests in Oxford.

Neutralizing antigen

In Edinburgh the antigen for the neutralizing antibody assays was produced in RK 13 cells (obtained from Moredun Research Institute, Edinburgh) grown in

199 medium (Glaxo) with 10% calf serum. Monolayer cultures in Roux bottles were infected with a multiplicity of greater than one and the cells harvested when the cytopathic effect was complete. To harvest the antigen, the medium was discarded and the infected cells removed by glass beads in 5 ml. medium per Roux bottle. This cell suspension was treated at full power in an MSE ultrasonicator for 5 min. and the resulting clear fluid used as the antigen. Infectivity titrations were carried out according to the method of Peutherer & Smith (1966) in BHK 21 cells (Macpherson & Stoker, 1962). At Oxford primary amnion cells were infected with a dose of virus which produced a cytopathic effect in every cell in 36–48 hr. The supernatant fluid was removed and centrifuged at 3000 rev./min. for 20 min. and the supernatant from this was removed for use. The infectivity of this supernatant was determined by inoculation of 0·1 ml. amounts of ten-fold dilutions into tubes of primary amnion tissue cultures.

Complement-fixing antigen

This was prepared in Edinburgh as for the neutralizing antigen except that the infected cells were removed from the glass in 5 ml. distilled water per Roux bottle. Suspensions were ultrasonicated and then centrifuged at 32,600 ***g*** for 30 min. in a Spinco model L ultracentrifuge using the SW 39 or SW 25 head to remove particulate matter. The supernatant was used as the complement-fixing antigen. Different batches of antigen were standardized against a reference herpes antiserum before use. It was found with both the reference herpes antiserum and the Colindale standard complement-fixing antiserum that the optimal dilution for the detection of herpes antibody was 1/32. For the purposes of this paper, the most concentrated antigen employed (a 1/4 dilution) will be referred to as the CF (conc.) antigen and the optimal dilution of antigen as the CF (opt.) antigen.

At Oxford, the optimum dilution of antigen was used in complement-fixation tests. The antigens used were kindly supplied by the Standards Laboratory, Central Public Health Laboratory, Colindale, and by Dr C. A. Ross at Ruchill Hospital, Glasgow (Grist, Ross, Bell & Stott, 1966). Sera were tested at 1/4–1/16. Those with titres of less than 1/8 were tested for neutralizing antibody.

Sera

All the Edinburgh sera were collected in the year 1965. They came from the following sources:

(1) 131 fourth-year medical students (of these 52 were Scottish born and educated).

(2) 75 probationer nurses from the Royal Infirmary, Edinburgh. These young women were bled during their period of preliminary training before they had been on duty in the wards.

(3) 376 specimens of acute phase serum submitted for diagnostic purposes to the Virology Unit, Bacteriology Department, Edinburgh University. These sera came from people living in the south-east of Scotland.

(4) 159 sera submitted to the Virology Unit of the Congenital Abnormalities

Research Unit of Edinburgh University. These specimens were from women of 16–43 years of age attending ante-natal clinics.

(5) 123 acute phase sera from patients admitted to the City Hospital, Edinburgh, and to the Royal Hospital for Sick Children, Edinburgh. These sera had all been submitted for virological investigation at the Wellcome Laboratory, City Hospital.

The Edinburgh age–antibody survey was carried out on 710 people comprising groups (3), (4) and (5) above together with 52 Scottish-born and -educated medical students. The Oxford sera were collected over the period 1962–65 and were from ante-natal women (18), medical neurological patients (95) and British-born medical students (52).

All sera were inactivated at 56° C. for 30 min. after dilution.

Neutralizing antibody assay

In Edinburgh this was carried out according to the accelerated adsorption technique of Peutherer & Smith (1966) using BHK 21 cells. Initially all sera were tested at dilutions of 1/8–1/256 against 100 TCID 50 of the virus. To save time, this was later altered to a screening technique, the dilutions tested being determined by the previously obtained complement-fixing antibody titre. Thus sera which had a complement-fixing antibody titre of $< 1/8$ were screened at dilutions of 1/8 and 1/16 in the neutralization test, while sera which were positive by the complement-fixation test were screened at dilutions of 1/128 and 1/256. Any sera which did not give an end-point at these dilutions were tested subsequently with the full range of dilutions, viz. 1/8–1/256. In Oxford the neutralizing antibody was assayed by a plaque reduction test. This test was carried out on all sera which did not show complement-fixing antibody. A 1/4 dilution of the inactivated serum was mixed with 50–100 plaque-forming units of virus. After 45 min at room temperature, equal volumes were inoculated to human amnion tissue cultures in test tubes. Sera which almost completely inhibited (90 % reduction) plaque formation were considered as positive.

Complement-fixing antibody assay

The Oxford sera were tested in agglutination trays (WHO) (Andrews & McDonald 1955) using an optimum dose of either of two antigens, $2\frac{1}{2}$ HD 50 of Burroughs Wellcome complement and overnight fixation at 4° C. In Edinburgh, the sera were tested as above except that 2HD 50 complement was used and a full checkerboard titration carried out on each serum. Doubling dilutions of the inactivated sera up to 1/1024 were prepared in Wasserman tubes before transfer to WHO Perspex plates. Standardized antigen at dilutions of 1/4–1/128 was used. Controls of each dilution of serum and antigen were included in each plate along with the appropriate complement controls.

RESULTS

Sera from 131 fourth-year Edinburgh medical students (average age 22 years) and 75 student nurses (average age 19 years) were examined for the presence of complement-fixing and neutralizing antibodies to *Herpesvirus hominis*. The results in Table 1 show that, in Edinburgh, only 40 % of the medical students and 48 % of the nurses have any detectable antibody. In Oxford, where 52 British-born medical students were examined, only 36 % were found to have detectable antibody.

Table 1. *The incidence of neutralizing and complement-fixing antibodies in sera from medical students and nurses*

Subjects	No. examined	Both neutralizing and CF antibody	Neutralizing antibody only	CF antibody only	Percentage showing either antibody
Edinburgh					
Medical students	131	46	3	3	40
Nurses	75	30	3	3	48
Oxford					
Medical students	52	—	—	—	36

Table 2. *The incidence of neutralizing and complement-fixing antibodies in the various age groups of subjects examined*

		Neutralizing antibody		Complement-fixing antibody			
				CF (conc.) Ag.		CF (opt.) Ag.	
Age	Total	No. positive	% positive	No. positive	% positive	No. positive	% positive
0–2 months	11	11	100	9	82	6	55
3–5 months	12	9	75	4	33	2	17
6–11 months	16	3	19	5	31	2	13
1 yr.	22	8	37	9	41	5	23
2 yr.	22	6	27	7	32	5	23
3 yr.	20	7	35	8	40	5	25
4 yr.	11	4	37	3	37	3	27
5 yr.	21	10	48	10	48	9	43
6–9 yr.	29	16	55	14	48	13	45
10–14 yr.	57	20	35	18	32	12	21
15–19 yr.	75	52	69	52	69	39	52
20–24 yr.	127	83	65	82	65	67	53
25–29 yr.	68	51	75	53	78	49	72
30–39 yr.	82	69	84	68	83	56	68
40–49 yr.	43	38	88	37	86	31	72
50–59 yr.	36	26	72	29	81	22	60
60–69 yr.	29	28	97	27	93	18	62
70– yr.	29	28	97	27	93	19	66

Neutralizing antibody

The results of the antibody studies on the 710 sera from the Edinburgh area are shown in Table 2 and Fig. 1. Here a high incidence of neutralizing antibody (75–100 %) is found in children under 6 months. The incidence then declines to

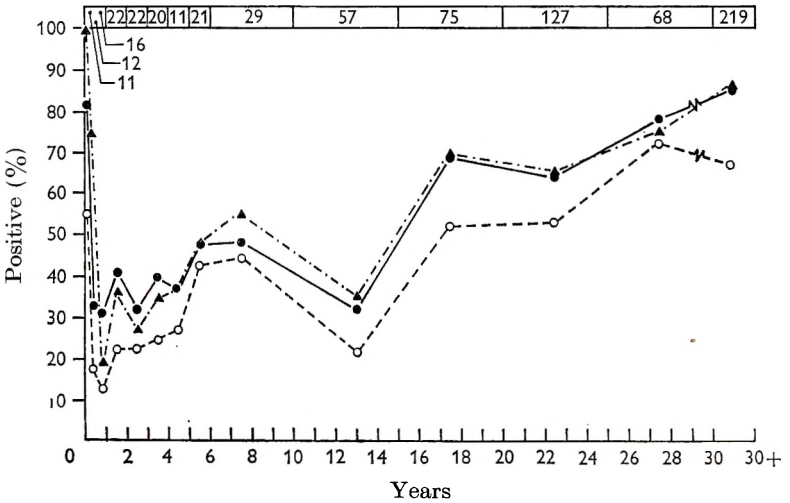

Fig. 1. The distribution of neutralizing and complement-fixing antibodies in the Edinburgh 1965 survey. ●——●, C.F. 1/4; ○— — ○, C.F. 1/32; ▲—.—▲, neutralizing antibody.

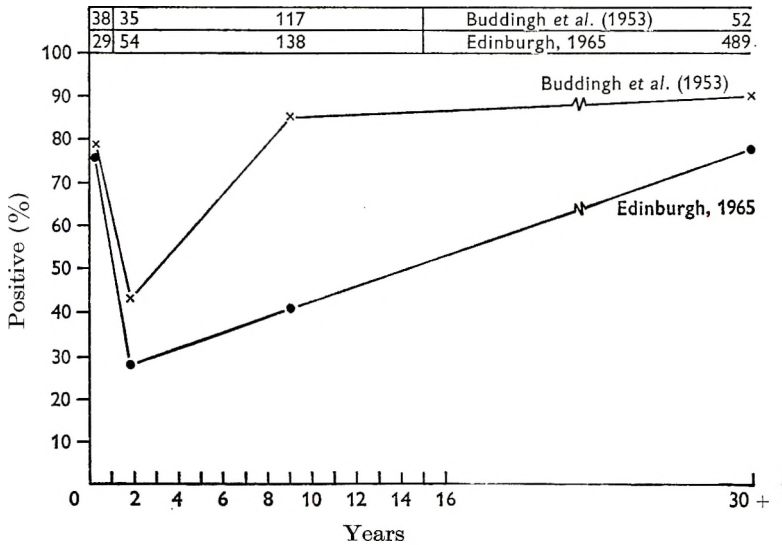

Fig. 2. Comparison of the incidence of neutralizing antibody in Edinburgh and New Orleans (Buddingh, Schrum, Lanier & Guidry, 1953).

19 % at 6–11 months, after which it rises slowly until 15–25 years when it is 65–69 %. Thereafter it increases to an ultimate 97 % in the over 60 age groups.

As the previous antibody surveys have all employed different age groupings, the

results of this survey have been regrouped according to the methods of Buddingh *et al.* (1953) and Yoshino *et al.* (1962). These are expressed graphically in Figs. 2 and 3.

Comparison of the Edinburgh survey with the New Orleans results in white children of Buddingh *et al.* (1953) shows that in the under 6-month group, the incidence of antibody was very similar (76 and 79 %). In subsequent age groupings, however, a decreased incidence was recorded in Edinburgh despite the greater

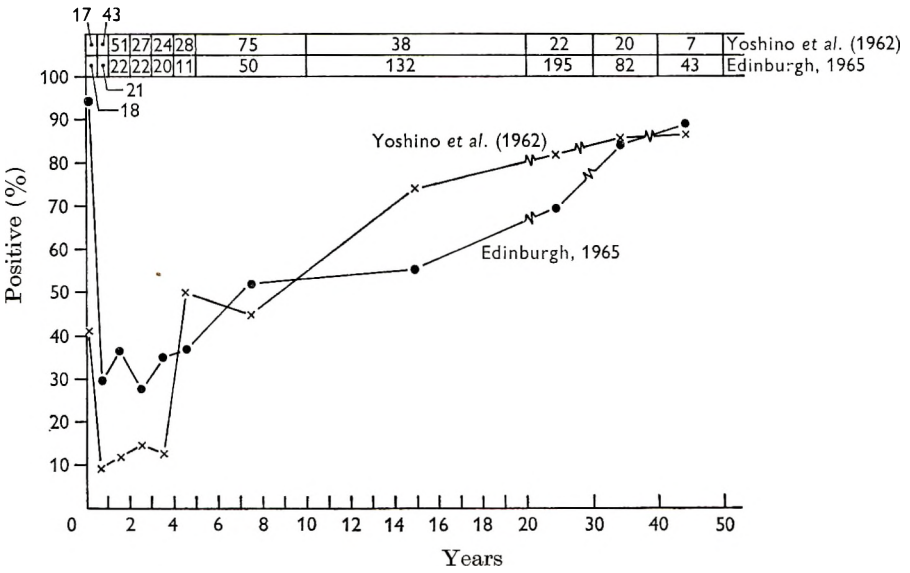

Fig. 3. Comparison of the incidence of neutralizing antibody in Edinburgh and Tokyo (Yoshino *et al.* 1962).

number of specimens examined. The decrease ranged from 15 % in the 7-month to 2-year group through 44 % at the 3–14 year group to 13 % in the over 14-year-olds. Figure 3 illustrates the comparison of the Edinburgh results with those of Yoshino *et al.* (1962) in Tokyo. Here it is noted that the results show a similar trend except for an increased incidence in Japan in the 10–19-year group. This may be a reflexion of the number of specimens examined in these age groups.

Complement-fixing antibody

The incidence of complement-fixing antibody in the Edinburgh sera is shown in Table 2 and Fig. 1, where the reaction to two different antigen concentrations is recorded. When the CF (conc.) antigen is used, the results obtained closely parallel those of the neutralization test except in the under 6-month group in which the incidence of neutralizing antibody is in excess of that of complement-fixing antibody. With the CF (opt.) antigen, the incidence of antibody is lower, especially in the younger and older age groups. In the 6-month–1-year and 1–2-year groups, the CF (opt.) antigen detected 18 % and 17 % fewer children with antibody compared with the CF (conc.) antigen while in the over-30-year age group the decrease was 19 %.

The complement-fixing antibody results were also regrouped to allow comparison with the results of Holzel *et al.* (1953) in England, Yoshino *et al.* (1962) in Tokyo and Halonen (1955) in Helsinki. These results are expressed graphically in Figs. 4–6.

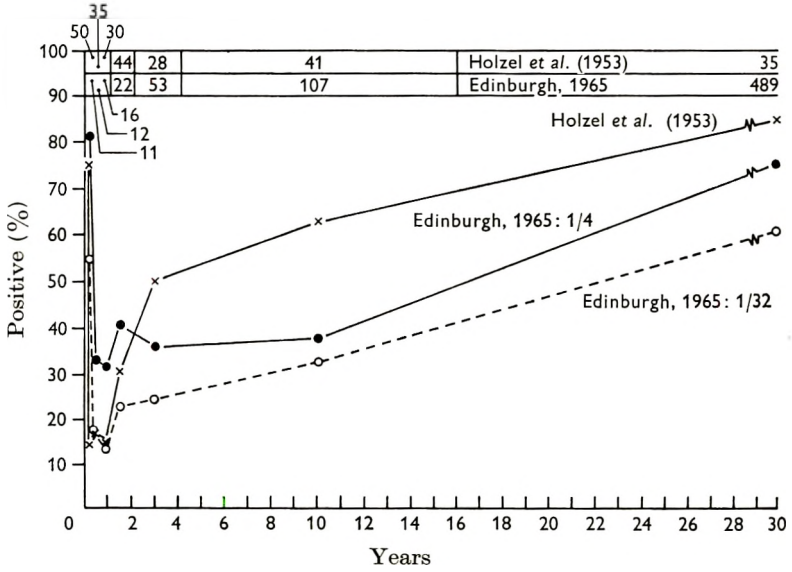

Fig. 4. Comparison of the complement-fixing antibody in Edinburgh and Manchester (Holzel, Feldman, Tobin & Harper, 1955).

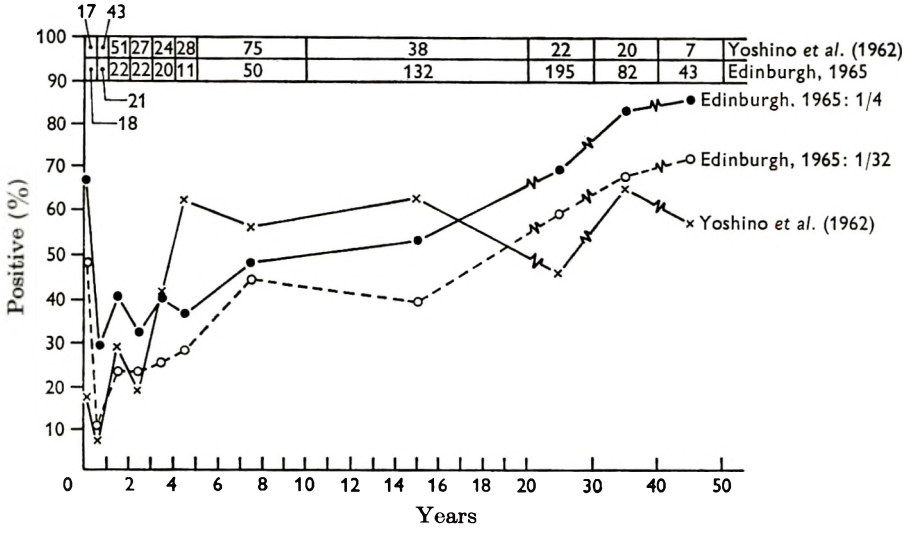

Fig. 5. Comparison of the complement-fixing antibody in Edinburgh and Tokyo (Yoshino *et al.* 1962).

Using the CF (opt.) antigen, the incidence of complement-fixing antibodies in the under-2-year group closely parallels that of Holzel *et al.* (1953) (Fig. 4), but at subsequent age groups there are fewer people with detectable antibody in 1965 (25, 30 and 24% respectively). While the CF (conc.) antigen detects a higher

proportion of positive sera in the under-2-year-olds, it still shows a lower incidence of antibody in the older age groups (14, 25 and 9% respectively) than that reported by Holzel *et al.* (1953).

Figure 5 shows that the use of the CF (opt.) antigen gives similar results to those of Yoshino *et al.* (1962) over the 4-month to 3-year age groups with an increased incidence when the CF (conc.) antigen is employed. The peak in the 4–5-year group in Japan is not reflected in the Edinburgh results and, in fact, up to the age of 20 years the Edinburgh results are lower than the Japanese ones. After

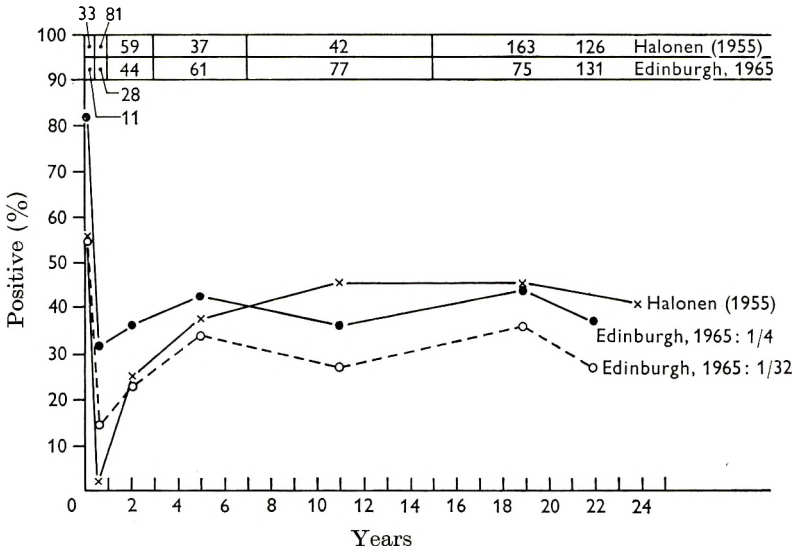

Fig. 6. Comparison of the complement-fixing antibody in Edinburgh and Helsinki (Halonen, 1955).

20 years, however, the incidence of herpes antibody increases in the Edinburgh survey and is in excess of that found in Japan. This may be a reflexion of the numbers in the groups and the techniques employed.

Halonen's (1955) results (Fig. 6) show a very similar trend to those obtained in Edinburgh except in the under-1-year-olds where again, although the Edinburgh CF (opt.) antigen results approach those of Halonen (1955), those of the CF (conc.) antigen give a higher incidence of antibody. In this case it should be noted that over the age of 15, the Edinburgh results for nurses and medical students were used, as they appeared to be more comparable to the conscripts and medical students cited by Halonen (1955).

DISCUSSION

Early workers showed the importance of socio-economic conditions on the incidence of *Herpesvirus hominis* antibodies in sera (Burnet & Williams, 1939). This observation has since been supported by evidence from several other sources. Thus Kibrick & Gooding (1965) in the U.S.A. found that while 84% 'ward service' patients and 62% of private patients had antibody, only 30–37% of college

undergraduates and young doctors possessed any antibody. Becker (1966) compared the incidence of antibody in three populations in Cape Town. He found that in the white population there was a lower incidence of antibody than in the coloured population, but that the Bantus had the highest incidence of antibody.

MacCallum (1959) found that within the 20–50 age group there was a 40–95% variation in the incidence of antibody found in females in four different communities. These observations are confirmed in Table 3 where it is noted that the Oxford and Edinburgh results for the over-50-year-olds are practically identical (87 and 89%), whereas those of the younger age groups lie within the 40–95% limits.

Table 3. *Comparison of the results of antibody studies carried out in Oxford and Edinburgh*

Oxford				Edinburgh			
Year	Age (yr.)	No.	Positive (%)	Year	Age (yr.)	No.	Positive (%)
1962	18–40	18*	66·65	1965	16–43	159*	84
1964	30–49	41†	73·9	1965	30–49	125‡	87
1964	50–69	54†	87·0	1965	50–69	65‡	89

* Antenatal.
† Medical Neurological Service.
‡ Either neutralizing or CF antibody detected.

In this survey, no attempt has been made to classify the people on a socio-economic basis, although it was thought that as the Edinburgh sera and the Oxford adult and antenatal sera were mainly from patients under National Health Service care, there should be no weighting towards the higher socio-economic groups. In addition, the sources of sera employed were very similar to those of previous workers. Acute phase sera submitted for viral diagnostic procedures were used, since such specimens should give a more accurate picture of the incidence of antibody in the general population than convalescent phase sera.

The question of the inclusion of all the nurses and medical students in the survey was considered. As these are both large groups it was felt that they might weight the survey unduly in their approriate sections. Another drawback was that a considerable proportion of the students were from countries other than Scotland, so only the 52 Scottish born and educated medical students were included in the appropriate age groups of the Edinburgh survey.

Apart from the difficulty of background of people included in a survey such as this, there are obvious difficulties in comparing the results of surveys carried out in different parts of the world, at different times, using a variety of antigens and techniques to estimate antibody. Different surveys have employed a number of age groupings, so that subsequent surveys may not always contain an equivalent distribution of people. This defect is obvious in the numbers of Edinburgh sera examined in the under-5-year group, but in this survey emphasis was placed on the 5–25-year group in view of the findings with the medical students and their close similarity to those of Halonen (1955). It will be noted, however, that the

neutralizing and CF (opt.) antigen results in the younger age groups are in agreement with previous findings despite the fewer children in the groups in this survey.

In view of the results of Ashe & Scherp (1963) who found, in neutralization kinetic studies with rabbit antisera, that the laboratory strain HF showed the least cross-reactivity among a number of strains of *Herpesvirus hominis*, a freshly isolated, wild strain of the virus was employed in this survey. In a subsequent publication, Ashe & Scherp (1965) failed to repeat their observation when human serum was used but they did emphasize the difficulty of establishing a base-line in this case. It was, therefore, decided to carry out a small preliminary comparison (unpublished results) using a number of wild strains of the virus isolated in the Edinburgh laboratory. When these were examined by the techniques employed in this survey no distinction could be made between the strain used and the others tested. Similar results were obtained in Oxford.

The close similarity of the incidence of the neutralizing antibodies with that detected by the CF (conc.) antigen suggests that the latter was an efficient antigen for the detection of herpes antibody. When the CF (opt.) antigen was employed, however, the incidence of positive sera was much lower in both the younger and older age groups. This would be expected in the younger age groups as some authors (Murray, Gaon, O'Connor & Mulahasanović, 1965; Grist *et al.* 1966) have reported the necessity of using a concentrated antigen for the detection of primary antibody in sera. The disparity in the older age group may be due in some measure to the quicker decay of the complement-fixing antibody. In this survey 75 % of children under 6 months, in whom the antibody would be largely maternal in origin, had neutralizing antibody, but only 35 % or 17 % had complement-fixing antibodies depending on the antigen employed.

The statement often found in textbooks that 90 % of the population over the age of 15 have neutralizing antibodies was taken from the work of Buddingh *et al.* (1953). This, though an accurate interpretation of the population studied in that paper, does not give a true picture of the situation pertaining today in some other communities in the U.S.A. and in several other countries. Buddingh *et al.*(1953) showed that 85 % of their white children in the 3–14-year age group had neutralizing antibodies, whereas only 41 % of the Edinburgh group had neutralizing antibodies in 1965. In the over-14-year-olds there is a less marked decrease in the incidence of antibody (90–78 %), but as the number of sera examined in 1965 was sufficiently large, it was possible to subdivide this age group. When this was done it was found that the incidence of neutralizing antibody did not exceed 70 % till after the age of 25. Dascomb *et al.* (1955) who, like Buddingh *et al.* (1953) also studied American citizens but from widely scattered areas, found in their series that the incidence did not exceed 70 % till after the age of 30. The sera examined were from air-force personnel and Dascomb *et al.* (1955) suggested that this lower incidence might be due to a high standard of hygiene.

The discrepancy in the incidence of neutralizing antibody in the Edinburgh survey between the 10–14-age group (35 %) and the 15–19-year age group (69 %) seems too great to be due entirely to chance differences in the socio-economic background of the 57 and 75 teenagers respectively on whom these observations

were made. The discrepancy might be correlated with the following clinical observations. First, Jones (1959) has shown that there are two main age groups for the onset of primary ocular herpes, viz. the under 10-year-olds and the 15–25-year age group. Secondly, in Sweden, Eilard & Hellgren (1965) found a steeply rising frequency of herpetic skin infection, in both men and women, after the age of 15 years. This incidence reached a maximum between 20 and 24 years. Lastly, Gold, Stewart & KcKee (1965) recorded a maximum incidence of herpes labialis in the 21–30-year age group. As there does not appear to be any published data concerning the long-term duration of the antibody response in young children following primary herpetic infection it is impossible to determine if this apparent increase in the incidence of herpetic infection in young adults is related to a decrease in the titre of circulating antibody or due to a greater degree of direct oral contact.

Stern *et al.* (1959) recorded the importance of herpetic whitlow as an occupational hazard of nurses, especially in neurosurgical units. These authors reported a 51 % incidence of antibody to herpesvirus in their nursing staff. In this survey, both in Oxford and Edinburgh, there is again a low incidence of antibody in nurses and medical students. A herpetic whitlow has been diagnosed in seven nurses and physiotherapists and one medical student in Oxford in the past 5 years. Thus it would appear that there is still considerable risk of herpetic infection in these young adults in the course of their professional duties.

The CF (opt.) antigen is probably more comparable with the complement-fixing antigens employed by previous workers, but despite using the results from the CF (conc.) antigen, a marked decrease in the incidence was found especially in the comparison with the results of Holzel *et al.* (1953). In the 5–14-year age group there is a decrease of 25 % in the number of people with antibody. Holzel *et al.* (1953) conducted their survey at least 12 years before the Edinburgh one so that children who were 5–14 years old in 1953 would be 17–26 years old in 1965. The incidence of antibody in this group of the 1965 survey was found to be 68 % of the 208 people with the CF (conc.) antigen and 53 % with the CF (opt.) antigen as compared with 63 % in the 1953 survey. Similarly, the 2–4-year age group of the Manchester survey, with a 50 % incidence of antibody, would be equivalent to the 14–16-year age group of the Edinburgh survey which shows an incidence of 53 % positive with the CF (conc.) antigen and 50 % with the CF (opt.) antigen in the 38 people studied.

It therefore appears that there has probably been a decrease in the incidence of herpetic infection in some communities in this country in children compared with 12 years ago, and it would be of interest to determine if this trend has continued in 10 years time. If this is so, there should be a reduction in the number of persons with detectable antibody in the over-30-year age group.

The recent findings of Kapsenberg (1964) and Ross, Subak Sharpe & Ferry (1965) of the antigenic cross-reaction between *Herpesvirus hominis* and varicella-zoster virus do not invalidate the findings of this survey. Acknowledging this cross-reaction, there still appears to be a decrease in the number of people with detectable antibody in certain age groups relative to the number 12 years ago. The

question of whether this antibody was due entirely to infection with *Herpesvirus hominis* or was a result of an anamnestic response to varicella virus applies to all the surveys to date.

This apparent decrease in the incidence of herpetic infection may possibly be explained by improvements in the social environment, especially in improved housing conditions with less overcrowding in homes, coupled with, perhaps, an increased awareness of simple hygienic requirements among greater numbers of the population. Similar factors were suggested by Yoshino *et al.* (1962) to explain the changing incidence of antibody which they found in Japan. Perhaps the similarity of the Edinburgh findings to those of Halonen (1955) also point to an improvement in hygienic conditions in Scotland.

If there is an increase in the proportion of people reaching adolescence with no detectable antibody to *Herpesvirus hominis* it is possible that primary infection of all types (of the skin, mucous membranes and brain) may be seen more often (cf. MacCallum, 1959; Stern *et al.* 1959) in this age group.

SUMMARY

Sera from 1029 individuals, 864 from Edinburgh and 165 from Oxford, have been examined for the presence of antibodies to *Herpesvirus hominis*. The results of the smaller Oxford survey did not reveal a higher incidence of antibody where direct comparison was possible with those from Edinburgh.

The incidence of both complement-fixing and neutralizing antibodies in the sera from 710 people in Edinburgh with ages varying from 1 month to 92 years was compared with the more complete of the earlier surveys, and in particular with that of Holzel *et al.* (1953) in Manchester, England. This comparison revealed a lower incidence of antibody in people under 25 in Edinburgh in 1965.

Results obtained with sera from medical students in Oxford and Edinburgh and from nurses in Edinburgh were in agreement with those of previous surveys. The low incidence of antibody in these young people emphasized the possible occupational risk of infection from patients and that primary herpetic infection might be encountered more frequently than before in teenagers and young adults.

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The occurrence of neutralizing and complement fixing antibodies in rubella

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Since rubella virus was isolated in 1962 neutralization tests in tissue culture have been used widely to detect antibody in human sera. Complement-fixing antigens have also been developed (Sever *et al.* 1965; Stern, 1965; Schell & Wong, 1966; Schmidt & Lennette, 1966*a*; 1966*b*) and are commercially available.

As in other virus diseases, rubella neutralizing and complement-fixing antibodies are not identical, the former being more persistant. For surveys therefore the neutralization test is more useful as an indicator of past infection, whereas the complement-fixation test is of value in studies of acute and of congenital rubella infection.

MATERIALS AND METHODS

RK 13 *cell cultures*

These cultures (Beale, Christofinis & Furminger, 1963) were grown at 37° C. in Pyrex Roux bottles using synthetic medium 199 (S.M. 199) containing 0·088 % sodium bicarbonate and 5 % calf serum. A mixture of 0·05 % trypsin and a 1/2500 dilution of versene in phosphate buffered saline solution A (Dulbecco & Vogt, 1954) was used to remove cells from the bottles. Pyrex tubes (16 × 150 mm.) were each seeded with 100,000 cells in 1 ml. growth medium and confluent cell monolayers were obtained after 3 days. At this stage the growth medium was replaced by maintenance medium consisting of S.M. 199 containing 0·176 % sodium bicarbonate and 1 % calf serum. The tubes were then rolled at 36·5° C. for 3 days before inoculation but no further medium changes were made. Calf serum was not inactivated at any time.

Virus for use in neutralization tests

The 'West Point' strain of rubella virus adapted to grow in RK 13 cells was used. Virus pools for the neutralization tests were prepared in RK 13 bottle cultures and stored in 1 or 2 ml. volumes at − 70° C. Titres of these pools ranged from $10^{3·0}$ to $10^{5·0}$ TCD 50 per 0·1 ml.

Neutralization test

The diluent was S.M. 199 containing 0·088 % sodium bicarbonate. Sera were not inactivated before testing. Serial twofold or fourfold dilutions of serum in 0·3 ml. volumes were mixed with equal volumes of 'West Point' virus diluted to contain

about 100 TCD 50 in 0·1 ml. After incubation in a warm air incubator at 36° C for $1\frac{1}{4}$ hr. the virus-serum mixtures were placed at 4° C. for 1–2 hr. Each virus-serum mixture in 0·2 ml. volumes was inoculated into each of two RK 13 tubes. A positive human serum of known titre was included in each test.

Tubes were read on the fourth and the sixth or seventh days after inoculation. Microfoci were not counted, but the degree of cytopathic effect was estimated by direct microscopy. The end-point in the antibody titration was taken as the highest dilution causing complete or almost complete inhibition of the cytopathic effect.

Titres are expressed as the initial serum dilution before mixing with virus.

Complement-fixing antigen

This was bought as a lyophilized 20 % cell pack of rubella-infected LLC-MK 2 cell cultures.

One ampoule of the antigen was reconstituted with 2 ml. veronal buffered saline pH 7·2 as a 1/2 dilution. This was stored in 0·1 ml. volumes at −70° C. until required for use when 0·1 ml. was thawed and diluted. On titration against a high-titre positive human serum the optimal dilution of the available batch was 1/24 but to ensure sensitivity in tests with unknown sera a 1/16 dilution was used, i.e. a further 1/8 of the stock dilution.

Complement-fixation test

A microtest five volume method adapted from Sever (1962) and Pereira, Pereira & Law (1964) was used, each unit volume being 0·025 ml. delivered with a calibrated dropping pipette. Sera were inactivated at 56° C. for 30 min. before testing. Serial twofold dilutions of serum were titrated against the 1/16 dilution of antigen in W.H.O. plastic plates. Complement, 1·8 to 2·0 MHD in 1 vol., was added to each virus–serum mixture. The plates were sealed with Sellotape, kept overnight at 4° C. and then held at room temperature for 10 min. before the sensitized cell suspension was added. The haemolytic system consisted of equal volumes of a 2 % suspension of sheep erythrocytes (standardized with the aid of a spectrophotometer) and a 1/100 dilution of haemolysin. The system was allowed to react for 15 min. at 37° C. before overnight storage at 4° C. Before they were added to the test the sensitized cells were warmed at 37° C. for 10 min. Two volumes of sensitized cells were added to each virus–serum-complement mixture and to all controls. The plates were again sealed with Sellotape and incubated at 36·5° C. for 1 hr.; for the first half hour they were shaken by hand at 10 min. intervals. Before the test was read the cells were left to settle for 3 hr. at 4° C. A known positive control human serum was included in each test.

Titres are expressed as the highest dilution of serum giving 50 % or more fixation with the antigen.

It is likely that complement-fixing antibody present early in the infant's life is maternal in origin and that the higher titres in older infants are developed by the child's own immune mechanism in response to the virus infection which continues after birth (Sever *et al.* 1966; Monif, Hardy & Sever, 1967). Mothers generally lose complement-fixing antibody by the time the child is 3 months old but in one instance (case 18) the mother's serum gave a positive reaction in the complement-fixation test when the child was 11 months old.

DISCUSSION

Work with experimental rubella in volunteers has shown that the presence of rubella neutralizing antibody at a titre of 1/4 or more prevents re-infection with the virus (Green *et al.* 1964, 1965; Schiff, Sever & Huebner, 1965). This indicates that detectable antibody and immunity are synonymous. It may be concluded from the surveys detailed here and from other published surveys (Givan *et al.* 1965; Sever, Schiff & Huebner 1964; Hutchinson & Thompson, 1965) that 80–95% of women in an industrial society are immune to re-infection with rubella virus. Local variations in the prevalence of immunity occur, as shown by the high degree of immunity in the Welsh mining community compared with the population of England and Wales as a whole. Similar variations have already been described (Sever *et al.* 1964; Report, 1967).

Unfortunately neutralization tests are laborious and expensive. The complement fixation test reveals antibody shortly after infection with rubella virus but is not suitable for detecting a rubella infection which occurred some years previously. Thus the complement-fixation test cannot replace the neutralization test in measuring immunity. The newly described haemagglutination inhibition test (Stewart *et al.*, 1967) should prove extremely useful for this purpose however since the findings tend to be similar to those of neutralization tests.

Because the positive complement-fixation test indicates recent rubella infection its occurrence in a mother and child shortly after the birth of the child will suggest rubella infection at least of the mother during pregnancy but not always infection of the foetus *in utero* because the antibody in the child at this early stage of life is largely maternal (Alford, 1965). Antibody in the child may either disappear in the first few weeks of life, in which case there was no intrauterine infection, or it may persist. If it persists this is interpreted by Alford (1965) as a gradual loss of maternal antibody (IgG) accompanied by an increase of the infant's own antibody, initially IgM then IgG, which remains indefinitely. The low titre of complement-fixing antibody found in very young infants, which correlates well with the mother's antibody titre, is residual maternal antibody. The high titres of complement-fixing antibody found in older children represent antibody synthesized by the child in response to rubella infection *in utero*.

The comparatively high neutralizing antibody titres found in mothers of children with congenital rubella may reflect a continuing antigenic stimulus resulting from persistence of infectious virus in the foetus during the last 6 months of pregnancy. Infants with congenital rubella commonly excrete virus in large

quantities even when there is neutralizing antibody in the circulation. This persistence of virus may account for the high antibody titres found in this group.

SUMMARY

Neutralization and complement-fixation tests to detect rubella antibody are described. Results of such tests show that between 80 and 95 % of adults in England and Wales have neutralizing antibody. The presence of complement-fixing antibody indicates recent infection with rubella virus.

Results are presented showing the development of neutralizing and complement-fixing antibody after rubella.

Antibody tests on children with congenital rubella and their mothers are detailed. It is noted that higher neutralizing antibody titres are found in this group than in the general population.

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The examination of samples infected with multiple salmonella serotypes

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INTRODUCTION

In diagnostic medical bacteriology the object is to isolate and identify a pathogenic organism. The result is used to aid diagnosis and control infection. In enteric fever or salmonella food poisoning one does not normally expect to find more than one pathogen in a sample, but it sometimes happens that several serotypes can be demonstrated in a patient (Hormaeche, Surraco, Peluffo & Aleppo, 1943; Harvey, Price, Davis & Morley-Davies, 1961; Taylor, 1960).

In the study of the epidemiology of salmonella infections, however, it is advisable to attempt to demonstrate the presence of all serotypes in a foodstuff as salmonella infections are spread by food. If this is not done, the epidemiological picture may be confused (Winkle & Rohde, 1958). Salmonellas can be isolated from a great variety of both human and animal foods and the latter are of interest because of the possibility that salmonella infections can be initiated and maintained in animals through contamination of their food-stuffs. The extent of this problem is debatable. Painstaking care is needed to succeed in isolating all, or nearly all, of the species present. An appreciation of the technical difficulty is important because one particular species might grow quickly in culture and appear to be the dominant pathogen, or even the only pathogen present. This paper sets out to discuss several methods of approaching the problem of multiple salmonella contamination which we have found helpful.

TECHNIQUES STUDIED

The methods examined were:-

(1) Use of colonial character to isolate certain serotypes.

(2) Picking of large numbers of suspicious colonies from a selective agar.

(3) Use of multiple subculture from selenite F broth (Harvey, 1957).

(4) Splitting the sample into several equal parts and selectively culturing each part in selenite F broth.

(5) Use of agglutinating sera to remove serotypes from a mixture in an orderly and premeditated manner (Harvey & Price, 1962).

(6) Use of a physical or chemical method to encourage the growth of one serotype rather than another. Here we should also consider the avoidance of a method that will effectively discourage the isolation of certain serotypes which grow less abundantly than the majority.

METHODS

1. *Use of colonial character to isolate certain serotypes*

Most salmonellas usually produce colonies on solid media which are indistinguishable from each other. A few serotypes, however, have colonies which *may* be characteristic on certain selective agars.

Such organisms are: *S. paratyphi B* (Schleimwall-Versuch, Müller 1910), *S. pullorum* (very small characteristic colonies on brilliant green MacConkey agar), *S. dublin* (small colonies on brilliant green MacConkey agar, normal-sized colonies on S.S. agar and deoxycholate citrate agar and often marked inhibition of growth on Wilson and Blair's agar). *S. typhi* sometimes, but not invariably, produces characteristic colonies on Wilson and Blair's medium and brilliant green MacConkey agar. The recognition of such minor degrees of differentiation can aid considerably in the search for these serotypes in difficult materials. When other serotypes are also present in such samples, all such assistance is extremely valuable. Colonial differences between certain salmonellas have been discussed in some detail by Stokes & Bayne (1957). The number of serotypes mentioned in this paper could be extended from our own experience.

Familiarity with the colonies of *S. pullorum* came with the weekly examination of many samples of Australian frozen egg in 1956 and 1957. We should hesitate to accept the implication that many medical bacteriologists are unfamiliar with the culture of this serotype (Lancet, 1965). Report (1958) records the fact that more than fifteen medical laboratories co-operated in the examination of egg products of various origins. From nearly 20,000 samples, 2414 strains of *S. pullorum* were isolated. We do not think that many infections in man are due to this serotype in South Wales and our close liaison with the local veterinary laboratory encourages us in this belief.

S. dublin has been searched for in the local abattoirs over the last decade and its frequent successful isolation is largely due to the recognition of its cultural peculiarities. On one occasion a single Moore's swab from an abattoir (Moore, 1948; Harvey & Phillips, 1961) produced colonies on brilliant green MacConkey agar (Harvey, 1956) of two salmonella serotypes: *S. dublin* and *S. typhimurium*. The colonial characters of the two varieties appeared distinctive, *S. dublin* colonies being much smaller than those of *S. typhimurium*. To test our ability to recognize *S. dublin* visually we picked 24 characteristic colonies for identification. Of these 20 were found to be *S. dublin*.

Polluted water supplies are examined weekly in Cardiff for salmonellas and we always incubate the brilliant green MacConkey plates to encourage slime-layer development on *S. paratyphi B* colonies. This usually allows differentiation between *S. paratyphi B* and other salmonellas growing on the same plate (Harvey, 1956). It also sometimes allows the recognition of slime-layer-negative strains of *S. paratyphi B*. The isolation of such organisms is of interest (Wilson & Miles, 1964*a*). The majority of isolations of *S. paratyphi B* from abattoirs and meat markets in South Wales have been slime-layer-negative. All the isolations from Indian crushed bone have also failed to produce slime layers. One recent culture of *S. paratyphi B*

from meat and bone meal said to be of British origin was slime-layer-positive. Outbreaks of gastro-enteritis in man caused by slime-layer-negative *S. paratyphi B* have been recorded recently (Report, 1964: Kingsley Smith & Thomas, 1966) and the subject of animal to human spread has been discussed in the past (Medical Officer, 1951). *S. paratyphi B* is not to be regarded as entirely host-specific for man (Buxton, 1957).

Sewage is so frequently contaminated with many salmonellas that a search for a single variety can be difficult and time-consuming. If foci of *S. paratyphi B* are being surveyed in the sewage of a large city (Harvey & Phillips, 1955) then the slime layer test is a necessity.

Although the colonial characters of other salmonellas are often indistinguishable from each other, this is not always the case. Sometimes on Wilson and Blair's medium different serotypes can be picked by differences in colony size and appearance. This has also been noted by Arnold (1956). This phenomenon can be useful in the examination of sewage, meat, natural water and animal feeding-stuffs.

2. *Picking and identifying large numbers of suspicious colonies from a selective agar*

This is the most usual method of examining specimens containing multiple serotypes. It has worked well in the hands of Hormaeche *et al.* (1943) and Juenker (1957). It is also applicable to the isolation of multiple phage-types of *S. paratyphi B* from a single patient (Sloan, Wilson & Wright, 1960).

Table 1. *Serotype distribution in 50 colonies picked from Wilson and Blair medium*

Serotype	no. of colonies identified as this serotype
S. anatum	11
S. bronx	1
S. jodhpur	5
S. karachi	10
S. kirkee	9
S. oranienburg	13
S. richmond	1

In our earlier work on the isolation of salmonellas from imported crushed bone we used this method, picking colonies to the water of condensation of small agar slopes in bijou bottles. These slopes were incubated for 6 hr. when the turbidity in the water of condensation was usually sufficient for slide agglutination to be attempted with H agglutinating sera. The picks were made direct from Wilson and Blair plates with a straight platinum wire. Contamination with other organisms was seldom experienced. A single example suffices as illustration. A subculture was made to Wilson and Blair agar from crushed bone cultured in selenite F broth; 50 colonies from the selective agar were isolated and identified. The serotypes found are shown in Table 1. Had only ten colonies been picked, only four serotypes would have been identified.

3. *The use of multiple subculture from selenite F broth*

The picking and identification of 50 colonies is not necessarily an impossible task even in routine practice. Yet the typing of this large number of colonies at a single subculture time may not be able to reveal the presence of some of the serotypes in the sample. The identification of fewer colonies from plates inoculated at different subculture times from the same enrichment broth may often give a more satisfactory result. This technique was first used as a routine in 1955 in the examination of swabs placed in open floor drains in bakeries (Harvey & Phillips, 1961). Topley & Fielden (1922) pointed out that, in an ordinary broth culture from a specimen of faeces, various bacteria succeed one another as the dominant viable organisms. In selenite F broth inoculated with material containing several salmonella serotypes, the ratio of one serotype to another is a function of the time of subculture (Harvey, 1965). Dixon (1959) found in a study of competitive growth of mixed coliform cultures in nutrient broth, that the organism inoculated in smallest numbers increased proportionately after 18 hr. incubation but there was no tendency for it to become dominant.

The type of result to be expected from the multiple subculture technique is best illustrated by a single experiment. Naturally infected crushed bones after 1 hr. incubation in broth at 37° C. were separated from the supernatant fluid. This fluid was then diluted to approximately 50 ml. with tap water, and 50 ml. double strength selenite F broth was added. The 100 ml. of fluid was incubated at 43° C. (Harvey & Thomson, 1953). Subcultures were made to Wilson and Blair's medium at 5, 18, 24, 48 and 72 hr. The plates were incubated at 37° C. for approximately 48 hr. and examined. The plate inoculated from selenite broth at 5 hr. incubation

Table 2. *Interdependence of serotype proportion and subculture time from enrichment medium*

Serotype	Percentage of 48 colonies picked at subculture times				
	5 hr.	18 hr.	24 hr.	48 hr.	72 hr.
S. derby	0	0	2	29	4
S. enteritidis (jena)	0	27	0	0	0
S. newport	0	0	0	10	0
S. oranienburg	0	15	17	4	6
S. reading	0	2	4	8	81
S. typhimurium	0	56	77	47	8

showed no salmonella-like colonies. All the other plates appeared positive and 48 suspicious colonies were picked from each to small agar slopes. The agar cultures, after incubation, were investigated and six serotypes were identified. The percentage (of 48 colonies) representing each serotype at the five subculture times is given in Table 2.

Obviously more serotypes could be isolated by multiple subculture than by relying on a single subculture time.

The technique finally chosen for routine practice was to pick 12 colonies from a plate representing each subculture time. Four subculture times were used. The

method was of great service in the earlier stages of our crushed bone investigation but has latterly been replaced by other techniques (4 and 5) which have been found more efficient.

4. *Splitting the infected sample into several equal parts and culturing each part in selenite F broth*

This technique is simply an adaptation of the first method of obtaining pure cultures from mixed cultures (Lister, 1878) and carries with it the same criticisms (Wilson & Miles, 1964*b*).

If a litre of water containing several salmonella serotypes is divided into ten equal parts and each part is cultured separately in selenite F broth, it is likely that the serotypes isolated may not be identical in each 100 ml. portion.

This method has been found to be most convenient for weekly examination of the River Taff, which drains a large area of Glamorgan county. With the pasteurization of egg products, overt human infections caused by salmonellas have declined in this region. There is still, however, a great deal of interesting latent infection not coming to the attention of the laboratory. As a measure of this hidden salmonellosis, we examine weekly 1 l. of water from the Taff, for salmonellas. The sampling site is carefully chosen and we believe that the isolations reflect the latent salmonella infection in part of our area. The results of some of these examinations are given in Table 3. Isolations from the meat market and abattoirs in Cardiff and from local infections in man of serotypes corresponding to those found in the river are given for comparison.

It will be noted that this technique is equally suitable for the isolation of individual serotypes and separate phage-types of *S. paratyphi B* and *S. typhimurium*. The method is particularly convenient for the routine examination of animal feeding-stuffs (Harvey & Price, 1967).

5. *The use of agglutinating serum to remove serotypes from a mixture in an orderly and predetermined manner*

This technique differs from those in the preceding sections in that the element of chance is partially eliminated. The bacteriologist is in control of the decision which serotypes to remove from his mixture and to some extent which serotypes he wishes to recover. We will therefore consider this technique in some detail.

The use of agglutinating serum in the selective isolation of salmonellas is not new. It was tried unsuccessfully by Loefler in 1906 and with greater success by Wassén (1930), Bailey & Laidley (1955) and Juenker (1957). The method was employed in a relatively simple form (Harvey, 1957) and was later developed for the examination of crushed bone fragments imported from India and Pakistan (Harvey & Price, 1962; Harvey, 1965).

The method of serological separation as used by Juenker (1957) involved the identification of one serotype from a selective agar. Numerous salmonella-like colonies were then picked to semi-solid agar containing antisera for phase I and phase II flagellar antigens of the serotype identified. If, on incubation, no spread through the soft agar occurred, it was assumed that all the colonies picked were of

the same serotype as that originally identified. Where, however, spread did occur the growth was examined for other antigens. In this study precautions were taken to avoid the criticism of production of serologically induced variants. We have

Table 3. *Isolations of salmonellas from consecutive samples of River Taff*

Figures in parenthesis after *S. paratyphi B* and *S. typhimurium* are phage-types. All dates are in 1966.

No.	Date (w.e.)	Serotypes and phage-types isolated	Corresponding isolations from man*	Corresponding isolations from meat and abattoirs*
1	23. iv.	*S. liverpool*, *S. reading* *S. paratyphi B* (1)	*S. liverpool*, 7. iv.	*S. oranienburg*, 28. iii.
2	30. iv.	*S. brandenburg*, *S. liverpool*, *S. reading* *S. minnesota*	*S. paratyphi B* (Taunton), 13. v.	*S. panama*, 3. v. *S. liverpool*, 16.v.
3	7. v.	*S. liverpool*, *S. paratyphi B* (1)	*S. schwarzengrund* 28. vi.	*S. paratyphi B* (1) 24. v.
4	14. v.	*S. brandenburg*, *S. oranienburg*, *S. paratyphi B* (1)	*S. brandenburg*, 25. vii	*S. panama*, 13. vi. *S. schwarzengrund*, 25. vii.
5	21. v.	*S. panama*, *S. paratyphi B* (1), *S. paratyphi B* (untypable)		
6	11. v.	*S. liverpool*, *S. reading*, *S. panama*, *S. oranienburg* *S. paratyphi B* (1)		
7	18. vi.	*S. tennessee*, *S. typhimurium* (32), *S. paratyphi B* (1)		
8	25. vi.	*S. indiana*, *S. panama*, *S. schwarzengrund*, *S. typhimurium* (1 var. 5), *S. typhimurium* (32)		
9	30. vi.	*S. schwarzengrund*		
10	9. vii.	*S. liverpool*, *S. reading*, *S. schwarzengrund*		
11	16. vii.	*S. oranienburg*, *S. panama*, *S. schwarzengrund*, *S. paratyphi B* (1), *S. paratyphi B* (Taunton)		

* Date of isolation shown after serotypes.

employed this technique in a prolonged examination of 50 g. of crushed bone. During the course of the examination 617 suspicious colonies were picked to agar slopes and twelve different subgenus I salmonellas and four different subgenus III salmonellas were identified. One subgenus I serotype gave trouble in that we were uncertain whether it was *S. reading* or *S. saintpaul*. There were 114 such cultures whose antigenic formula was doubtful. All 114 cultures were examined by the phase change method of Harvey & Price (1961), but the H agglutinating sera added to the semi-solid agar in the pasteur pipette included both phase I factor

eh and Phase II factor 5. Of the 114 cultures introduced into the pipettes, 113 were immobilized and representatives of these were shown to be *S. reading*. The single culture that migrated through the soft agar was identified by the salmonella reference laboratory as *S. saintpaul* (1, 4, 5, 12: eh–1, 2).

To test further the specificity of separation we introduced a mixture of *S. typhimurium* and *S. agama* into the distal end of a pipette containing 0·15 % nutrient agar mixed with H Phase I, factor i, and H Phase II, factor 2, agglutinating sera. Incubation overnight at 37° C. resulted in growth to the upper surface of the serum-agar column. This surface growth was plated and after incubation of the plate only colonies of *S. agama* were identified. The experiment was repeated with *S. poona* and *S. bristol*. No difficulty was encountered in the serological separation of these two antigenically similar organisms. *S. enteritidis* and *S. dublin* were also separated from each other by this method. The manipulation of the agar filled pipettes has already been described (Harvey, Mahabir & Price, 1966).

Experiments were then conducted to demonstrate that a very small minority of one serotype could be separated from a different serotype dominant in a mixture. For convenience of observation a sucrose fermenting salmonella (Dixon & Curtis, 1960) was used as one of the pair of competing serotypes and a suitable indicator plating medium was employed. The result was invariable: the minority species could easily be separated from the dominant species.

Further observations showed that mixtures of many serotypes could be progressively separated into their constituent parts and each salmonella species isolated and identified. Later we found it possible to emulsify the entire bacterial growth present on a selective agar and to use this dense suspension as test material for examining for multiple serotypes. At first, like Wassén (1930), we added selenite to the semisolid agar + serum mixture, but subsequently this was found unnecessary and undesirable. The mere passage of a mixed growth of salmonellas and other organisms through a column of 0·15 % nutrient agar increased the ratio of salmonellas to other organisms present. (Harvey *et al.* 1966).

The following technique was finally adopted; for clarity an actual examination will be described. A 15 g. sample of crushed bone was placed in an 8 oz. wide-mouthed screw-capped jar. Sufficient nutrient broth was added to cover the bone fragments and the jar and contents were incubated at 37° C. for 24 hr. Double-strength selenite F broth was then added equal in volume to the bone fragments + broth. The jar was incubated in a 43° C. water bath for 24 hr. The enrichment broth was subcultured to Wilson and Blair's medium and this was incubated at 37° C. for 48 hr. Freshly poured Wilson and Blair was used prepared according to the formula of de Loureiro (1942). After incubation the plate showed many salmonella-like colonies with typical surrounding sheen. A colony was removed to a nutrient agar slope which was incubated for 24 hr. The Wilson and Blair plate was put on one side. The colony subcultured to agar was identified as *S. poona* (13, 22: z–1, 6). The entire remaining growth on the Wilson and Blair plate was wiped off with a sterile throat swab and emulsified in 0·2 ml. peptone water. Two loopfuls (2 mm. diameter) of H phase I, factor z, serum and two loopfuls of H phase II, factor 6, serum were added to 20 drops (20 × 0·02 ml.) of 0·15 % nutrient agar.

A pipette identical with that described by Harvey *et al.* (1966) was filled with this serum–agar mixture and the suspension from the Wilson and Blair plate was sucked into the distal bulb of the pipette underneath and in contact with the lower surface of the soft agar. This type of pipette was very easily prepared. An ordinary pasteur pipette was taken and the barrel heated in a bunsen flame at a point $\frac{1}{2}$–$\frac{3}{4}$ in. proximal to the junction of the neck and barrel. When molten, the glass was pulled out into a robust capillary 5 in. long. The capillary distal to the bulb so formed was cut off $\frac{3}{4}$–1 in. below it. The lower end of the filled pipette was sealed and the whole was incubated at 37° C. for 24 hr. Growth was now visible at the upper surface of the soft agar in the barrel. This growth was subcultured to a second Wilson and Blair plate which was incubated at 37° C. for 48 hr. The second plate was treated like the first and the process was serially repeated until no further serotypes were isolated. From the 15 g. of bones, *S. poona*, *S. gaminara*, *S. london*, *S. reading* and *S. hvittingfoss* were isolated one after the other. In practice 4 × 15 g. of bones were examined in this way simultaneously. This involves a combination of methods 4 and 5. In one sample of 4 × 15 g. of bones twenty different serotypes were found. Part of this work has already been published (Harvey & Price, 1962). It should be noted that none of the previously undiscovered serotypes reported in that publication was isolated by the serological method. The final list of serotypes isolated is given in Table 4.

It is worth noting that *S. typhimurium* was the sixth commonest serotype in this list and that 18 % of samples were infected with this organism. Similarly a relatively high percentage of specimens of fish meal imported into Holland were found to contain *S. typhimurium* by Jacobs, Guinée, Kampelmacher & van Keulen (1963). We think this point is worth emphasis. Indian and Pakistani material does not appear to be a constituent of animal feeding-stuffs owing to the danger of anthrax infection (Davies & Harvey, 1953, 1955). It does, however, raise the possibility that *S. typhimurium* may be more commonly present in animal feeding-stuffs than was previously thought. The search for a specific serotype in a sample is probably a question of adapting the serological technique so as to favour the isolation, or of adapting the present method of fluorescence microscopy to the same end. Crushed bone from India and Pakistan is a complex material for study. Almost 100 % of samples are infected with salmonellas. Salmonella counts are high (Smith, 1960) and many serotypes are present in each sample. The serological technique can, however, be used routinely for the examination of other samples such as polluted water, and swabs from abattoirs and from table surfaces and machinery in wholesale-meat premises. In such specimens the number of serotypes present is usually not more than two and the process is therefore much simpler.

6. *Use of two cultural techniques each designed to favour the isolation of separate salmonella serotypes*

The salmonella group is culturally inhomogeneous. *S. choleraesuis* is best isolated by means of a fluid or solid brilliant green medium. *S. typhi* will not grow at 43° C. but in our hands has shown itself to be resistant to 0·8 % selenite (Harvey, 1957; Harvey & Price, 1964). *S. pullorum* grows poorly at temperatures above

40° C. (Stokes & Bayne, 1957), but is well isolated from selenite F broth incubated at 37° C. and subcultured to brilliant green MacConkey. Such cultural idiosyncrasies have to be taken into account in the investigation of specimens containing these organisms especially if they are mixed with other salmonellas more easily cultured. Two separate cultural techniques may have to be used in such instances.

Table 4. *Salmonellas isolated from crushed bone imported from India and Pakistan*

Subgenus I			Subgenus I			Subgenus I	
Serotype		Times isolated	Serotype		Times isolated	Serotype	Times isolated
S. senftenberg		22	*S. kandla*		4	*S. havana*	1
S. anatum		21	*S. saintpaul*		4	*S. huvudsta*	1
S. newport		20	*S. seigburg*		4	*S. karachi*	1
S. cubana		19	*S. worthington*		4	*S. kirkee*	1
S. reading		16	*S. sp* (unidentified)		4	*S. lansing*	1
S. poona		14	*S. adelaide*		3	*S. marylebone*	1
S. typhimurium:			*S. derby*		3	*S. montevideo*	1
type 1 b (4a)	1		*S. enteritidis* (jena)		3	*S. newington*	1
type 22	1		*S. magwa*		3	*S. simsbury*	1
type 1 a (U57)	1		*S. muenchen*		3	*S. singapore*	1
type 2 (11)	1		*S. onderstepoort*		3	*S. taksony*	1
type U 131	1	14	*S. paratyphi B*:			*S. waycross*	1
type 1	1		type 1, var. 12	1		*S. urbana*	1
type 2c (14)	2		type 1, var. 3	1	3	Percentage of samples positive for subgenus I	99
untypable	3		untypable	1			
untyped	3		*S. stanley*		3		
S. gaminara		13	*S. telhashomer*		3		
S. butantan		12	*S. adamstown*		2		
S. cerro		12	*S. alachua*		2		
S. richmond		12	*S. champaign*		2		
S. tennessee		12	*S. godesberg*		2		
S. kentucky		10	*S. grumpensis*		2		
S. oranienburg		10	*S. jodhpur*		2		
S. westhampton		10	*S. matopeni*		2		
S. chester		9	*S. pomona*		2		
S. give		9	*S. sandiego*		2		
S. hvittingfoss		8	*S. schwarzengrund*		2		
S. bredeney		7	*S. treforest*		2		
S. london		7	*S. virchow*		2		
S. minnesota		6	*S. charity*		1		
S. meleagridis		5	*S. chingola*		1		
S. bere		4	*S. chittagong*		1		
S. bronx		4	*S. dublin*		1		

Subgenus III	
Serotype	Times Isolated
26:23–30	4
26:23–21	2
26:26–25	2
9a, c:29–31	1
16:22–31	1
20:24–28	1
29:24–31	1
29:33–21	1
30:23–31	1
30:27–28	1
Percentage of samples positive for subgenus III	15

In this laboratory we examine weekly specimens of stream water for *S. typhi*. Occasional samples are contaminated with *S. typhimurium*. We therefore culture the material in double strength and four times strength selenite F broth (Harvey & Price, 1964). The former medium encourages the growth of *S. typhimurium*, the latter that of *S. typhi*. Similarly when we were examining samples of Australian frozen egg, it was found that *S. pullorum* was best isolated at 37° C. and *S. typhi-*

murium at 43° C. Both procedures were therefore used. *S. dublin* is also a serotype whose cultural peculiarities have to be recognized. It is best searched for on deoxycholate citrate agar incubated at 37° C. or on S.S. agar incubated at 40° C. (Livingstone, 1965). These two plates are always used in the culture of abattoir specimens thought to contain this organism. The same samples are also cultured on Wilson and Blair's agar which effectively reveals other salmonella serotypes.

DISCUSSION

This paper may seem to overemphasize technical detail. To a practical bacteriologist, it is difficult to accept this as a valid criticism. Sir Almroth Wright prefaced his work on *The Technique of the Teat and the Capillary Glass Tube* (1912) with a quotation from Carl Ludwig—'Die methode ist alles'. The quotation is still apt today when standardization of technique is held to be of paramount importance. Method standardization was recently discussed by Hilbert (1966) and the danger of rigidity of technique was noted. As both the present authors were trained by early workers in Sir Almroth Wright's laboratory, the influence of his book on our technical approach is not unnatural.

In this paper we have recorded various methods which have been found valuable over a period of almost twenty years. During that time we have become familiar with a great many members of the salmonella group, and most Public Health Laboratory Service laboratories in England and Wales have had similar experience.

It is difficult to put forward an optimum technique for the examination of samples containing multiple serotypes. To some extent the time available for the examination is the deciding factor. Where this is short, method 4 is the most convenient. Where the full range of serotypes in a material is being investigated and time is not important, a combination of methods 4 and 5 is best. Where less complex material is examined, method 5 is the one of choice. Methods 2 and 3, though effective, are rather tedious. We seldom employ these techniques today. As already indicated, method 6 is suitable for a restricted class of sample where two serotypes are present with distinctive cultural characters.

It might be thought that the serological technique described here could be criticized on the grounds that serologically induced variants might be produced. This type of criticism is always difficult to counter, but if it were valid, we should expect our serotype isolations from Indian crushed bones to differ somewhat from serotypes actually isolated in the Indian subcontinent. Reference to the literature will show that this is not so (Ganguli, 1958; Sharma & Singh, 1961; Agarwal 1962).

SUMMARY

Attention is drawn to the confused epidemiological picture experienced in investigating outbreaks of salmonellosis due to multiple serotypes. Public Health Laboratories are often faced with the examination of specimens containing several serotypes and in this paper six techniques are described which have been found useful in dealing with this problem. The choice of technique depends on the time available for the examination and the epidemiological importance of obtaining an

accurate result. A preference is expressed for an immuno-isolation technique, in which the bacteriologist is in technical control of the isolation of specific serotypes. The method is possibly open to objection on the grounds of serological induction of new serotypes, but we should regard this danger as slight in the majority of samples examined.

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