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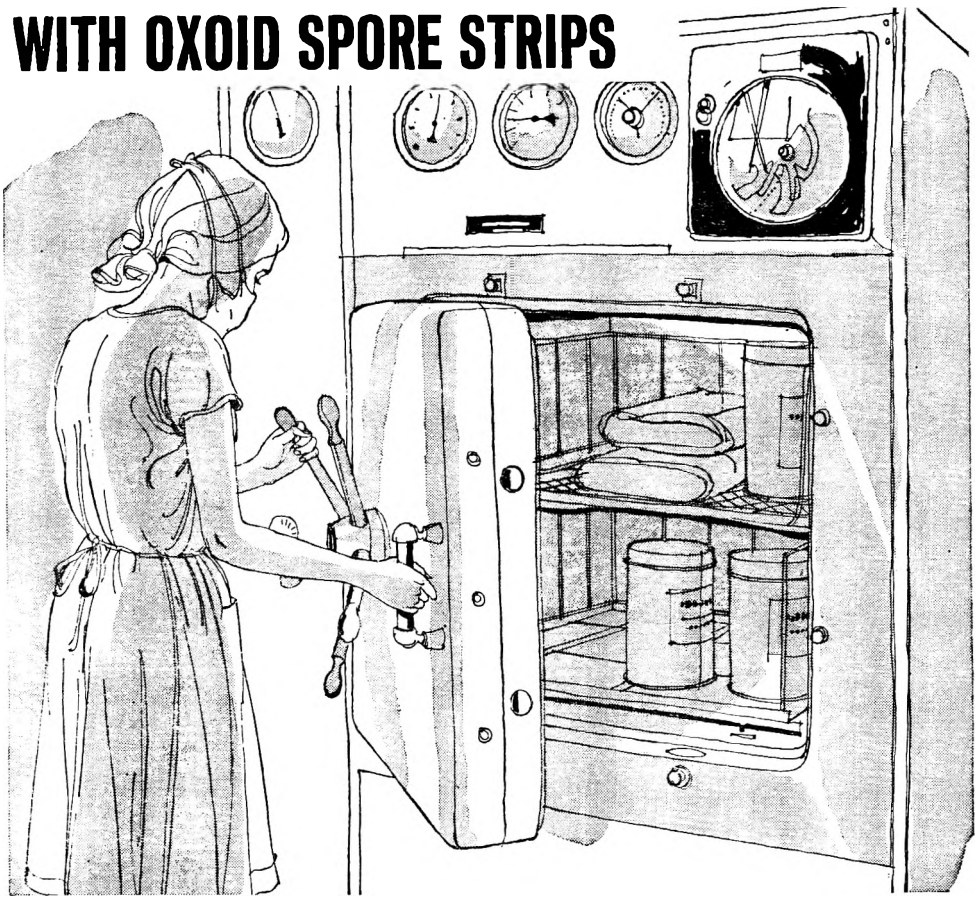

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Isolation of a variant strain of foot-and-mouth disease virus (type O) during passage in partly immunized cattle

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(*Received* 16 *April* 1966)

Evidence of the existence of different subtypes within the seven principal types of foot-and-mouth disease (FMD) virus was described by Hyslop, Davie & Carter (1963). Recently we reported the isolation of an immunologically distinct variant strain of virus of type SAT 1, which evolved when strain Tur. 323/62 was passaged serially through thirty-four partly resistant cattle (Hyslop & Fagg, 1965). The variant was distinct from all the other subtype strains of type SAT 1 against which it was tested, and these observations provided experimental support for theories on the mechanism by which new subtypes might arise during outbreaks of FMD in the field.

The present report records a similar experiment, using a virus strain of type 0, in which a new variant evolved after fewer passages than were required for the isolation of the variant strain of type SAT 1.

MATERIALS AND METHODS

Virus

FMD virus of type O (strain Israel 1/63), isolated during a recent epizootic, was inoculated into susceptible cattle at Pirbright and subsequently was passaged serially in further cattle which had been partly immunized with a vaccine prepared from virus of the homologous strain. The serial passage followed the procedure described by Hyslop & Fagg (1965) and the sequence is shown in Table 1.

Vaccine

The groups of cattle were vaccinated with one or several graduated doses of inactivated vaccine prepared by the method described by Morrow, Hyslop & Buckley (1966). A concurrent experiment demonstrated that the vaccine (batch 6405) was of high potency.

Experimental procedure

The source and maintenance of cattle, the methods used for titration of virus and for the serological tests were the same as the procedures described in the previous report (Hyslop & Fagg, 1965).

Table 1. *Passage of FMD virus of strain Israel* 1/63 *in partly immunized cattle*

Virus passage no.	Animal no.	Vaccination history (doses)	SVNT of sera against virus of: Original strain	SVNT of sera against virus of: EQ 33 strain	Virus inoculated: Source	Virus inoculated: Dilution	Clinical result of inoculation
A	EQ 90	Unvaccinated	N.A.	N.A.	Field	10^{-1}	T, 4F
	EQ 91	Unvaccinated	N.A.	N.A.	Field	10^{-1}	T, D, 4F
B	EQ 92	Unvaccinated	N.A.	N.A.	Tongue EQ 90/91	—	T, 4F
	EQ 93	Unvaccinated	N.A.	N.A.	Tongue EQ 90/91	—	T, M, 4F
1	EP 72	1 × 2·0 ml.	1/45	1/32	Tongue EQ 90/91	10^{-2}	T, 1F
	EP 73	1 × 2·0 ml.	N.A.	N.A.	In contact	—	
1*a*	EQ 76	1 × 15 ml.	1/90	1/45	Tongue EQ 90/91	10^{-2}	T
2	EP 74	1 × 2·0 ml.	N.A.	N.A.	Tongue EP 72	10^{-1}	T, 2F
3	EP 75	1 × 2·0 ml.	N.A.	N.A.	Tongue EP 74	10^{-2}	T, D, 4F
4	EP 76	1 × 5·0 ml.	1/90	1/45	Foot EP 75	10^{-3}	T, 1F
5	EP 77	1 × 5·0 ml.	1/6	1/6	Tongue EP 76	10^{-4}	T, 4F
6	EP 78	1 × 5·0 ml.	1/90	1/90	Tongue EP 77	10^{-5}	T, 2F
7	EP 79	1 × 5·0 ml.	1/32	1/22	Tongue EP 78	10^{-6}	T, D, 4F
—	EP 80	1 × 10 ml.	—	—	Tongue EP 79	10^{-7}	No reaction
8	EP 80	—	N.A.	N.A.	Tongue EP 79	10^{-6}	T, 4F
—	EP 81	1 × 10 ml.	—	—	Tongue EP 79	10^{-7}	No reaction
9*a*	EP 81	—	1/128	1/90	Tongue EP 80	10^{-6}	T, 1F
—	EP 82	1 × 10 ml.	—	—	Tongue EP 81	10^{-7}	No reaction
10*a*	EP 82	—	1/45	1/32	Tongue EP 81	10^{-6}	T, 3F
11*a*	EP 83	1 × 10 ml.	N.A.	1/6	Tongue EP 82	10^{-6}	T, 1F
—	EP 84	1 × 15 ml.	1/2048	1/355	Tongue EP 83	10^{-7}	No reaction
—	EP 84	—	—	—	Tongue EP 83	10^{-6}	No reaction
—	EP 84	—	—	—	Tongue EP 83	10^{-5}	No reaction
9	EQ 28	s.i. + 1 × 15 ml.	1/178	1/90	Tongue EP 80	10^{-6}	T, 1F
10	EQ 29	s.i. + 1 × 15 ml.	1/45	1/16	Tongue EQ 28	10^{-6}	T, 4F
—	EQ 30	s.i. + 2 × 15 ml.	—	—	Tongue EQ 29	10^{-6}	No reaction
11	EQ 30	—	1/1400	1/128	Tongue EQ 29	10^{-4}	T, 2F
12	EQ 31	2 × 15 ml.	1/90	1/45	Tongue EQ 30	10^{-4}	T, 1F
13	EQ 32	2 × 20 ml.	1/178	1/90	Tongue EQ 31	10^{-5}	T, 3F
14	EQ 33	2 × 20 ml.	1/355	1/128	Tongue EQ 32	10^{-5}	T, 1F
15	EQ 90	Recovered 34 days after passage A	1/512	1/128	Tongue EQ 33	$10^{-1\cdot3}$	T
15	EQ 91		1/2800	1/512	Tongue EQ 33	$10^{-1\cdot3}$	T
—	EQ 92	Recovered 33 days after passage B	1/1400	1/355	Tongue EQ 33	$10^{-1\cdot3}$	No reaction
—	EQ 72	1 × 15 ml. challenged, 35 days convalescent	1/355	1/90	Tongue EQ 32	$10^{-1\cdot3}$	(TE)
16	EQ 73		1/1024	1/256	Tongue EQ 91 (passage 15)	$10^{-1\cdot3}$	T

s.i., Sensitizing inoculation; SVNT, virus neutralizing titre of serum; T, primary vesicles on tongue; D, lesion on dental pad; M, secondary vesicle on muzzle; 1F–4F, secondary vesicles on 1 or more feet; N.A., result not available; TE, erosive lesion on tongue.

RESULTS

Clinical results in cattle

Passage of virus in vaccinated cattle

Groups of cattle were vaccinated subcutaneously with strain Isr. 1/63 vaccine in doses of 2·0 ml., 5·0 ml., 10·0 ml. or 15·0 ml. Other animals, some of which had been sensitized during innocuity tests of FMD vaccines, were vaccinated with either one or two doses of 15·0 ml. or with two doses of 20·0 ml.

In a concurrent experiment, a single 15·0 ml. dose of the vaccine protected all of ten steers (EQ 70–79) against generalization of lesions after challenge by inoculation of the tongue with the homologous virus.

Virus of the field strain Isr. 1/63 was passaged in the tongues of steers EQ 90 and EQ 91. A filtrate of the harvested material had a titre of $10^{6\cdot8}$ ID 50/ml. when titrated in two cattle by the method of Henderson (1952). One of these animals died as a result of generalized FMD.

On the 21st day after vaccination, a virus filtrate (diluted to contain $10^{3\cdot0}$ ID50/ml.) was inoculated into the lingual mucosa of steer EP 72. Vesicles appeared on the tongue after about 40 hr. and the overlying epithelium, harvested under general anaesthesia, was found to have a virus titre of $10^{9\cdot0}$ mouse ID50/g. Steer EP 73, vaccinated at the same time as steer EP 72 and housed in contact, did not develop FMD. Material from steer EP 72 was used to continue the serial passage in vaccinated steers shown in Table 1.

To ensure that the strain was capable of generalizing in the presence of antiserum and was not merely being passaged mechanically, virus harvested from the foot lesions of the third animal in the series was used for the 4th passage.

Because of individual variations in immunizability (Hyslop, 1966), a smooth 'gradient' of serum-virus neutralization titres (SVNT) was not established but the majority of the animals in the latter part of the series possessed SVNT which might have been expected to prevent generalization of infection to the feet if no change had occurred in the antigenic constitution of the virus strain.

Between the 2nd and 7th passages the dilution of the inoculum was increased tenfold at each passage but, when an attempt was made to passage the strain at a dilution of 10^{-7} for the 8th serial passage, steer EP 80 failed to react. On the following day, however, inoculation of the same virus suspension diluted only 10^{-6} resulted in infection and lesions generalized to all feet. Probably as a result of the balance between the infectivity of the inoculum and the SVNT of the recipient, by the 11th passage it was necessary again to decrease the dilution of the inoculum. The last two passages in vaccinated cattle were made at a dilution of 10^{-5}.

Table 2 indicates that the amount of virus in the inoculum exerted little influence on the titre of the resultant vesicles but the high SVNT of 1/355 at the 14th passage probably reduced the virus titre about 100-fold; if a change in antigenic structure had not occurred, this SVNT might well have been sufficient to inhibit the development of lesions completely. Infection generalized after each successful passage in the vaccinated cattle.

Reinfection of recovered cattle

After the 14th passage in semi-resistant cattle a 1/20 suspension of triturated tongue epithelium from the last animal (steer EQ 33) was inoculated at five sites in the lingual mucosa of each of the two steers EQ 90 and EQ 91; these animals had been infected with field virus at the start of the experiment. Although the animals had recently recovered from experimental infection, EQ 91 developed

Table 2. *Relationship between dilution of inoculum, antibody titre and titre of virus in tongue lesions of semi-resistant cattle infected with FMD virus*

Passage no.	Dilution of inoculum	SVNT (against original strain)	Virus titre of tongue epithelium (mouse ID 50/g.)
1	10^{-2}	1/45	$10^{9.0}$
3	10^{-2}	N.A.	$10^{9.5}$*
4	10^{-3}	1/90	$10^{10.0}$
6	10^{-5}	1/90	$10^{10.0}$
7	10^{-6}	1/32	$10^{10.5}$
10	10^{-6}	1/45	$10^{9.1}$
12	10^{-4}	1/90	$10^{9.5}$
14	10^{-5}	1/355	$10^{7.5}$

* Titre of epithelium from foot: $10^{5.0}$.
N.A. Result not available.

one and EQ 90 two small lesions at the site of inoculation. The same inoculum failed to cause lesions in steer EQ 92, although its SVNT against virus of the original strain was intermediate between those of steers EQ 90 and EQ 91. Similar material from the penultimate animal of the vaccinated group, when inoculated into the tongue of steer EQ 72 (vaccinated and then challenged 35 days previously in the course of the concurrent vaccine potency experiment), evoked an erosive lesion on the tongue which progressed to a shallow ulcer. A 1/20 suspension of tongue epithelium from the second reaction of the recovered steer EQ 91, when inoculated into the tongue of steer EQ 73 (vaccinated and challenged during the same experiment as EQ 72), resulted in the development of a single small vesicle of about 3 mm. diameter.

Serum-virus neutralization tests

All cattle were bled for serum immediately before experimental infection. The results of serum-virus neutralization tests against virus of the original strain and against the variant strain isolated from the last steer of the vaccinated group are shown in Table 1.

At the start of the experiment a SVNT of 1/90 against the original strain was associated with protection against that strain (steer EQ 76). Later in the series much greater titres were found in animals which failed to resist generalization.

The mean antibody titre of all serum samples was 1/190 with virus of the original strain and 1/68 with virus of the strain isolated from steer EQ 33 at the end of the serial passage. This indicates that the two strains of virus are different in antigenic structure.

Complement fixation tests

Part of the vesicular epithelium harvested from the tongues of infected cattle was triturated and used as antigen in complement fixation tests employing the stock type antisera prepared by the World Reference Laboratory (W.R.L.). Other samples of epithelium were used in tests for which strain-specific antisera were prepared.

Typing tests

Epithelium from the tongue vesicles of the donor steers fixed complement strongly in the presence of W.R.L. antiserum of type O. When the strain was sub-inoculated into steers EQ 92 and EQ 93, complement fixation tests on epithelium again revealed only virus of type O; one of these animals (steer EQ 93) died, however, and material from the heart lesions found at necropsy fixed complement strongly with W.R.L. serum of type O and also showed slight fixation with stock sera of types Asia 1 and C. Thereafter, traces of fixation with sera of type C were detected in epithelium samples collected from the partly immunized cattle at the 1st, 8th, 9th and 13th pasages. The O/C ratio was determined for virus of the 1st, 2nd, 3rd, 9th, 12th and 14th passages. The cross-reactions were not consistent and there was no evidence of a trend towards greater similarity to type C. Virus from the 1st passage fixed a trace of complement in the presence of antiserum of type Asia 1 but no complement was fixed with serum of this type by virus isolated after the last passage.

Table 3. *Cross-fixation products of FMD virus samples isolated during passage in partly immunized cattle*

	Virus from animal no.:			
	EQ 90	EP 72	EP 77	EQ 33
EQ 90	1·00	1·00	0·54	0·32
EP 72	1·00	1·00	0·52	0·38
EP 77	0·54	0·52	1·00	0·44
EQ 33	0·32	0·38	0·44	1·00

Tests using strain-specific sera

Guinea-pig antisera were prepared with virus derived from the tongue lesions of steers EQ 90 and EQ 91, the unvaccinated cattle, also from steer EP 72, the first partly immune animal, and from steers EP 77 and EQ 33, the 5th and the last of the partly immune cattle, respectively.

The cross-fixation products of the various serum-virus pairs are shown in Table 3. A considerable degree of variation was found after five passages of strain Israel 1/63 in vaccinated cattle. Although the change between the 5th and the 14th passages was smaller than that detected after the first five passages, the difference between the original strain and that isolated after the 14th serial passage was very great.

DISCUSSION

At the first passage of strain Israel 1/63 in partly immunized cattle, the virus was just able to generalize to a single foot of a steer which possessed a SVNT of 1/45 but it failed to generalize in an animal whose SVNT was 1/90. At the 4th and 6th passages, generalization occurred in cattle having SVNT of 1/90 and, by the 9th passage, the virus was able to generalize despite SVNT of 1/128 and 1/178, respectively. This developing ability to generalize suggests that the virus had adapted to some extent to its new and adverse environment in semi-resistant cattle; the change apparently occurred quite rapidly. Additional evidence of an early modification of antigenic structure is afforded by a decrease in the cross-fixation product (C.F.P.) between the first and the fifth passages (Table 3). However, the difference in C.F.P. between the first and second passages was not significant and this renders it less probable that the change resulted solely from differential inhibition within an initially very heterogeneous virus population.

By the 14th serial passage the C.F.P. with virus of the first passage clearly indicated that a distinct strain had evolved. Furthermore, when the cattle were infected, each possessed antibody against virus of the vaccine strain and the mean SVNT of all serum samples was 1/190: the lower mean SVNT of 1/68 of the same serum samples, when tested against virus isolated from the 14th animal in the passage series (steer EQ 33), provides still further evidence of antigenic variation in the virus.

Recovered cattle generally have been found to resist reinfection by intramuscular inoculation of large amounts of virus of the homologous strain for periods exceeding two years; inoculation of the same virus into the lingual mucosa will cause small primary lesions some months after clinical recovery, though such lesions are usually limited in number and generalization of infection does not occur. Inoculation of virus of heterologous subtypes by the latter route may evoke both primary and secondary vesicles after shorter periods. By the 14th serial passage in partly immunized cattle, the variant of strain Israel 1/63 had altered sufficiently to cause small primary vesicles when inoculated into the tongues of steers EQ 90 and EQ 91. It is noteworthy that reinfection of these animals was achieved only 34 days after their original infection with strain Israel 1/63. At the time of reinfection, steer EQ 91 possessed a SVNT of 1/2800. Steer EQ 92 failed to react to the same inoculum. Despite a SVNT of 1/1024, steer EQ 73 (vaccinated and then challenged with strain Israel 1/63, to which it reacted 35 days previously) developed primary vesicles when it was inoculated again with virus from the second infection of steer EQ 91.

After experimental infection, the virus titre of the epithelium covering the vesicles of semi-resistant cattle was found to be very high and this titre was affected little either by the dilution of the inoculum or by the titre of antibody in the subject's serum. Samples of saliva from these cattle were highly infective and, undoubtedly, such cattle would have been capable of disseminating FMD under field conditions.

In spite of the great infectivity of the lesions caused by the first passage of strain

Israel 1/63 in vaccinated steers EQ 72 and EQ 76, a third vaccinated steer (EP 73), housed in contact with them, failed to develop clinical signs of FMD and was apparently protected against contact infection by a single 2·0 ml. dose of vaccine. At the end of the experiment, however, this animal was found to have developed a SVNT of 1/708 against virus of the original strain; it must be assumed, therefore, to have experienced an inapparent infection. A similar inapparent infection in vaccinated steer EP 84 evoked a SVNT of 1/2048, a value greatly in excess of vaccinal titres, and this was sufficient to protect the animal against infection when the time arrived for it to be inoculated with virus of the 11th serial passage. Inapparent infections of this nature have been observed in other experiments in which vaccinated cattle have been exposed to infection by contact. It is possible, therefore, by leaping ahead of any emergent strains, that 'parent' strains in the field may occasionally reinforce vaccinal immunity to a level at which dissemination of emergent strains may be inhibited or blocked completely. If such a sequence of events occurs frequently in the field, the emergence of variants may depend upon a delicate balance of numerous factors and the outcome (i.e. dominance or otherwise of the variant) may be largely fortuitous.

The results of the present investigations show certain contrasts with those of a previous experiment employing strain Tur. 323/62 of type SAT 1, but the differences may be attributable to modifications of technique as much as to differences in the strains of virus. During serial passage of strain Israel 1/63, the inoculum was diluted progressively from the 2nd to the 7th passage, whereas in the earlier experiment the inoculum was diluted progressively only after the 26th passage. Furthermore, in the second experiment the total number of serial passages to which strain Israel 1/63 was subjected was lower than that for strain Tur. 323/62. Nevertheless, the results of these investigations confirm our previous observations and demonstrate that antigenic lability is not confined to FMD virus of a single strain or type. It is also evident that, under the adverse conditions of serial propagation in semi-resistant hosts, such lability may be manifested after only a few (less than ten) passages. The difference between the mean SVNT of all the post vaccinal sera, when tested against virus of the original and the variant strains, was less in the case of the type O strains (mean SVNT 1/190 and 1/68, respectively) than for the strains of type SAT 1 (mean SVNT 1/80 and 1/4). Similarly, although complement-fixation tests may be quantitatively less reliable than neutralization tests, the C.F.P. of the original and the variant strains was slightly greater (C.F.P. 0·325) after 14 passages of strain Israel 1/63 than after 34 passages of strain Tur. 323/62 (C.F.P. 0·27). It is probable, therefore, that strain Israel 1/63 was modified to a somewhat lesser extent than was strain Tur. 323/62.

The original sample of bovine tongue epithelium received from the field outbreak, from which strain Israel 1/63 was subsequently isolated, not only fixed complement strongly in the presence of W.R.L. antiserum of type O but also showed very slight cross-fixation with antiserum of type C and a trace of cross-fixation with antiserum of type Asia 1. Complement fixation tests on virus isolated from the saliva of reacting cattle also showed traces of cross-fixation with antiserum of type C. At the outset it appeared possible that passage of the virus in cattle

whose serum contained antibody strongly inhibitory to virus of type O might evoke a change towards type C, and complement fixation tests revealed slight cross-fixation with type C intermittently throughout the experiment. It is noteworthy that FMD virus of type C has not been recorded in Israel. However, at no stage in the experiment did the passaged strain show a greater affinity for type C than for type O and the principal changes occurred only in antigenic constituents specific for type O. Whether or not subsequent serial passage in cattle partly immunized against the *variant* strain might lead eventually to the evolution of virus of one of the other types remains a matter for conjecture.

New strains have been recovered during passage of FMD virus in monolayer cultures of porcine kidney cells in the presence of strain-homologous antiserum (Hyslop, 1965).

SUMMARY

Foot-and-mouth disease virus of type O (strain Israel 1/63) was passaged serially fourteen times in cattle previously vaccinated with increasing doses of formol-treated vaccine of the homologous strain. Primary and secondary lesions developed in the majority of the animals. Virus from the 14th passage, which proved to be capable of reinfecting animals only 34 and 35 days after infection with the original strain, differed from the original strain in complement-fixing properties and in sensitivity to antiserum. These changes were of a degree indicative of subtype variation.

Virus titres in the saliva, in the tongue vesicles and in the foot vesicles of partly resistant cattle were very great and would have constituted a danger to other stock under field conditions.

The experimental data confirm previous observations on the infection of partly resistant cattle and on the isolation of variant strains during passage of FMD virus in tissue cultures containing specific antiserum.

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Toxoplasmin sensitivity: subnormality and environment

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INTRODUCTION

There is some conflict of opinion regarding the importance of congenital toxoplasmosis as a cause of mental subnormality. Along with choroidoretinitis, hydrocephaly, microcephaly and convulsions, gross mental retardation is one of the classical signs of congenital toxoplasmosis, but the complete syndrome is extremely rare.

Thalhammer (1962) found that the incidence of positive dye test in grossly retarded infants without other signs of congenital toxoplasmosis was 20 % higher than that found in normal controls of the same age. He suggested that these children were suffering from what he termed 'oligosymptomatic toxoplasmosis'. More recently Remington (personal communication) claimed to have shown that a small but significant proportion of very young retarded children investigated by him owed their condition to toxoplasma infection. In contrast, Burkinshaw, Kirman & Sorsby (1953), Cooke & Derrick (1961), Fleck (1963) and Labzoffsky, Fish, Gyulai & Roughley (1965) all failed to find any evidence that toxoplasmosis was a significant cause of subnormality in the populations they studied. Although my dye-test survey of subnormals in hospital (Robertson, 1965) showed 20 % more positives among child defectives than among normal children the incidence was as high in mongols as in epileptics and other subnormals. Since the chromosomal anomaly responsible for mongoloid development seemed unlikely to be a result of protozoal infection, I concluded that the excess of dye-test positives among subnormal children was probably due to postnatally acquired infections and not to congenital disease. It seemed possible that as a result of some environmental or social factor severely subnormal children might be exposed to a greater risk of acquiring infection than were normal children.

The present investigation was undertaken in an attempt to locate such a factor.

Owing to the grave shortage of beds in the area it is difficult to secure early admission of defectives to hospital. If home care is possible subnormal children have to remain with their parents. Waiting-lists are very long, and can only be by-passed if home care is completely lacking or if serious behaviour problems render it impracticable. Consequently the children in hospitals for the subnormal are a highly selected group, many of whom were admitted for reasons similar to those which cause normal children to be taken into County Council children's homes. In order to see whether it was exposure to such conditions which had caused the high incidence of infection in the children in hospital I decided to test

a group of children admitted to children's homes in addition to a group of subnormals who were living with their parents and attending day training centres run by the County Council.

METHODS AND MATERIALS

Parental consent for a toxoplasmin skin-sensitivity test was sought in respect of all subnormals attending day training centres and in respect of children admitted to the children's homes run by the County Council of Lincoln, Parts of Lindsey. Those for whom consent was obtained were tested by intradermal injection of 0·1 ml. of an antigen supplied by Eli Lilly Inc., the antigen being a dilute sterile preparation made from peritoneal exudate of infected mice. The results were read on the third day, when an area of induration exceeding 7 mm. in diameter was read as a positive test.

The antigen and technique were the same as those used previously to survey subnormals residing in hospitals (Robertson, 1965).

RESULTS

The results of skin tests on subnormals under the age of 20 attending training centres and those in hospital are shown in Table 1. The total figures for patients under the age of 20 show the same incidence (12 in 120 = 10 %) in training centre cases as that found among hospital cases (23 in 233 = 9·9 %).

Table 1. *Toxoplasmin skin tests of subnormal patients*

	Hospital			Training centre			Total				
Age	No.	No. +ve	% +ve	No.	No. +ve	% +ve	No.	No. +ve	% +ve	S.E. of diff.	Diff./S.E.
1–9	53	6	11·3	50	1	2·0	103	7	6·8	4·97	1·87
10–17	139	11	7·9	54	11	20·4	193	22	11·4	5·1	2·45
18–19	41	6	14·6	16	0	0·0	57	6	10·5	9·0	1·62
Total	233	23	9·9	120	12	10·0	353	35	9·9	—	—

The standard error of the difference in incidence between training centre cases under 10 years of age and those between 10 and 17 is 6·25, and diff./S.E. = 2·94.

The standard error of the difference in incidence between training centre cases aged 10–17 and those aged 18–19 is 10·0, and diff./S.E. = 2·04.

When the distribution by age is examined in greater detail, however, one finds that patients aged between 10 and 17 years attending training centres show a significantly higher incidence than do older or younger training-centre patients or hospital patients of the same age.

It seems likely that these differences may have resulted from selection. The most severely subnormal cases tend to remain insufficiently mature to attend training centres until after the age of 10, and either remain at home or enter hospital. They may attend training centres during their early 'teens' but are more likely than the higher-grade defectives to become unmanageable and require hospital care in late adolescence.

At the other end of the scale there are some children of higher intellectual ability who are educated in special schools up to the age of 16, but subsequently fail to adjust to employment, and are ascertained as subnormals and admitted to training centres at about the age of 18. The middle age range of training-centre patients would therefore tend to contain a higher proportion of low-grade patients than would the younger and older groups. A further selection factor could have resulted from the restriction of testing of training-centre patients to those whose parents consented, since consent given on admission permitted the testing of all the hospital patients.

Table 2. *Comparison of toxoplasmin skin sensitivity between subnormal and normal children in County Council care*

	Subnormals			Normal children in care			Total		
	No.	No. +ve	% +ve	No.	No. +ve	% +ve	No.	No. +ve	% +ve
1–9	103	7	6·8	19	0	0·0	122	7	5·75
10–17	193	22	11·4	52	1	1·9	245	23	9·4
Total	296	29	9·8	71	1	1·4	367	30	8·2

The standard error of the difference in incidence between all subnormals under 18 and all children in care was 3·62. Diff./S.E. = 2·32.

Table 3. *Toxoplasmin skin tests on mongols and other subnormals*

		Mongols			Other subnormals		
Age		No.	No. +ve	% +ve	No.	No. +ve	% +ve
1–9	Training centre	15	1	—	35	0	—
	Hospital	9	2	—	44	4	—
	Total	24	3	12·5	79	4	5·1
10–19	Training centre	23	1	—	47	10	—
	Hospital	26	3	—	154	14	—
	Total	49	4	8·2	201	24	12·5
20–29	Training centre	7	1	—	18	0	—
	Hospital	12	2	—	134	37	—
	Total	19	3	15·8	152	37	24·4
30–49	Training centre	4	0	—	10	1	—
	Hospital	19	3	—	267	82	—
	Total	23	3	13·1	277	83	30·0

Despite these differences, it was plain that the high incidence of positivity was not confined to the hospital patients, and the overall incidence of positive skin test was about 10% in both hospital and training-centre patients.

The incidence among children admitted to County Council children's homes was very much lower, only one out of 71 such children being positive. Since children only remain in County Council care up to the age of 18, the subnormals above that

age were excluded from the comparison made in Table 2. There were 29 positive reactors among the 296 subnormals under the age of 18, an incidence of 9·8 % compared with the 1·4 % incidence among normal children in care. As the standard error is 3·62 the difference between these two groups was just significant. Adverse home circumstances leading to admission to children's homes do not therefore lead to increased exposure to risk of toxoplasma infection, and the high incidence in young subnormals in hospital was related to the fact and severity of mental defect rather than to their home environment.

Combining the hospital and training-centre populations, it was possible to compare 115 mongols under the age of 50 with 709 other subnormals under the same age. These two groups are compared in Table 3, which shows quite clearly that in childhood the incidence of infection in mongols is at least as high as, and probably higher than, that in other subnormals. The incidence of positive skin test in mongols remains at about 11 % irrespective of age, but that in other subnormals rises from 5 % in those below the age of 10 to 30 % in those over the age of 30. The pattern among subnormals other than mongols is that of gradual acquisition of infection from continued environmental exposure to risk, and that among mongols is more consistent with congenital infection.

DISCUSSION

Hitherto most authorities have considered it unlikely that the chromosomal anomaly responsible for mongolism was a result of infection. The recent demonstration by Stoller & Collmann (1965) that peaks of incidence of mongol birth follow 9 months after epidemics of infective hepatitis, however, suggests the possibility that infection with this virus may cause trisomy. Jirovec, Jira, Fuchs & Peter (1957) in Prague found 79 out of 94 mothers of mongol children to be positive to the skin test for toxoplasmosis. The incidence varied from 81 % in mothers under the age of 30 to 94 % in those over the age of 40, figures far higher than those found in other women of the same age. The fathers of 39 of the mongol children were also tested but only 10 (26 %) were positive. In view of this evidence of an association between maternal toxoplasmin sensitivity and mongol births, the possibility that toxoplasma infection might interfere with meiotic cell division and produce trisomy cannot be dismissed. Toxoplasma, like the viruses, is an obligatory intracellular parasite, and could have similar effects upon cells. Unless the ovum itself contained parasites, however, one would not expect the children to be infected, and it seems unlikely that an infected ovum would develop into a viable foetus. Although there is evidence (Langer, 1963; Werner, Schmidtke & Thomascheck, 1963; Remington, Newell & Cavanaugh, 1964; Beattie, 1964; Robertson, 1966) that some chronically infected women may be liable to abort repeatedly, there is no evidence that live-born children of chronically infected mothers are congenitally infected. In France more than 83 % of the antenatal sera are positive to the dye test (Desmonts, Couvreur & Ben Rachid, 1965) and in England 25 % are positive, but in both countries sera of children over the age of 6 months are rarely positive to the dye test. Desmonts, Couvreur, Allison *et al.* (1965) found no positive reactors among

86 babies aged 6 to 11 months, and I found only 4 out of the 171 children below the age of 5 to be positive (Robertson, 1965). It would seem that children of women with low stable dye-test titres are seldom congenitally infected, and that even if toxoplasma infection of the mother were the cause of some cases of mongolism, infection of the mongol children themselves is probably acquired postnatally. The finding that subnormals, whether resident in their own homes or in hospital, show the same high incidence of postnatally acquired infection suggests that it may be some personal habit or pattern of behaviour more common among grossly retarded than among normal children which leads to an increased risk of infection.

Until the mode of transmission of toxoplasma has been determined the nature of this habit must remain in doubt. The results of recent experiments by Hutchison (1965) suggest one possible explanation. Hutchison succeeded in infecting mice with toxoplasma by feeding them with an extract from the faeces of a roundworm-infested cat, which had eaten toxoplasma cysts. The extract remained infective despite prolonged storage in tap water at room temperature, and he believes that the parasites were protected within the ova of the roundworm *Toxocara cati*. Garden soil is often contaminated with the faeces of dogs and cats. Since subnormals take longer than normal children to learn clean habits they may more frequently eat with, or suck, dirty fingers.

SUMMARY

The incidence of positive toxoplasmin skin test among subnormals in hospital was compared with that in subnormals attending day training centres and among normal children taken into County Council children's homes. Twelve out of 120 subnormals below the age of 20 attending day training centres were positive to the test, and 23 were positive out of 233 subnormals of the same age tested in hospital.

There was only 1 positive out of 71 children under the age of 18 in the County Council children's homes, compared with 29 out of 296 subnormals of this age who were tested.

The incidence of positive skin test among 115 mongols under the age of 50 was about 11% in each age range, whereas that in 709 other subnormals rose from 5% in those below the age of 10 to 30% in those over the age of 30.

Although factors similar to those causing admission of normal children to County Council care often determine the admission of subnormals to hospital, the children in County Council homes did not share the high incidence of infection which was found in subnormals.

It is suggested that the majority of infections among subnormal children are postnatally acquired. Since the incidence was the same in subnormals living at home as among those in hospital it is suggested that some personal habit commoner among subnormal than among normal children exposes the subnormals to an increased risk of infection.

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An epidemiological study of *Salmonella* in a closed pig herd

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Various data concerning salmonella excretion in the faeces of groups of animals and birds have been described: in pigs (Hansen, 1964; Zagaevskii, 1962; Kampelmacher, Guinée, Hofstra & van Keulen, 1963; Gaugusch, 1963); in calves (Wormald, Creasby & Venn, 1965); in mice (Morello, Digenio & Baker, 1965); and in pigeons (Neilsen, 1965). Little work however has been done on analysing the results of repeated examinations over a period of time, particularly where it concerns the epidemiology of the infection inside a closed community of farm animals. The present paper describes an attempt at such an analysis.

An outbreak of clinical salmonellosis in a closed herd of hysterectomy-produced pigs during 1963/64 was described by Heard, Linton, Penny & Wilson (1965). It was decided to use this herd of pigs, in which salmonella infection had become established, for an epidemiological study. Previously only a brief reference was made to the subsequent pattern of salmonella excretion in the herd, and this is extended in this communication.

Regular faecal examinations from all occupied pens on the farm were made and the findings correlated with records of all animal movements and routine activities of the farm staff. By this means it was possible to establish the major factors responsible for the spread of salmonella infection within this closed community.

MATERIALS AND METHODS

The earlier examinations for *Salmonella* were carried out using individual rectal swabs of all the pigs on the farm. This was necessary since at that time some pens had communal dunging passages, which allowed cross-infection to occur, and hence the individual animal was the unit of examination. In order to limit cross-infection after the initial outbreak all pens were converted to individual dung disposal. Since each pen was now segregated, a small trial was carried out to compare the numbers of salmonellas that could be isolated from pen faecal samples, with those isolated from individual pig rectal swabs. The pen faecal samples were collected in polythene bags, the bag being extruded so that it formed a glove around the hand. In pens containing more than one pig, six or seven samples of freshly voided faeces were picked up from different parts of the same pen, care being taken to avoid treading on any area before taking the sample. This method was similar to that described by Kampelmacher *et al.* (1963)

and Guinée, Kampelmacher, Hofstra & van Keulen (1965). Rectal swabs of about 0·5 g. of faeces were incubated overnight in selenite broth and subcultured on deoxycholate citrate agar. Non-lactose fermenting colonies which grew after 24 and 48 hr. incubation were tested for urease production, to eliminate species of *Proteus*, and all urease-negative strains were tested biochemically and serologically to identify specific *Salmonella* serotypes. Strains of *Salmonella typhimurium* were phage-typed. The results are shown in Table 1 and a summary of the findings in Table 2.

Table 1. *A comparison of methods used for detecting* Salmonella

Pen no.	Salmonella isolations by: Pen faecal examination	Rectal-swab examination	Ratio of salmonella positive rectal swabs to total examined
1	–	–	0/13
2	–	–	0/11
3	–	–	0/9
4	–	–	0/1
5	–	–	0/7
6	–	–	0/10
7	–	–	0/10
9	–	+	3/11
10	+	–	0/1
11	+	+	1/1
12	+	+	1/1
13	+	–	0/1
14	–	–	0/8
15	–	–	0/7
16	–	–	0/1
17	–	+	6/11
18	+	–	0/1
Totals	5/17	4/17	11/104

Table 2. *Summary of data set out in Table* 1

Nature of isolation	No. of pens from which salmonellas were isolated by the different methods: Pen faeces examination	Rectal swab examination	Either method
Both methods	2	2	2
Faeces only	3	0	3
Rectal swab only	0	2	2
Totals	5	4	7
% of all pens infected	29·4	23·5	41·0

TRIAL RESULTS

Of the 104 rectal swabs examined, 11 were positive for *Salmonella* but despite the large number of examinations made only 4 pens were shown to be infected. By pen-faeces examination 5 pens were shown to be infected. The results of both methods did not show perfect correlation. Of the 7 infected pens, only 2 were positive by both methods, 2 by rectal swab examination only and 3 by the pen faecal sampling only. It is evident from this limited survey that neither method is significantly superior to the other, but the use of both methods gives higher incidence than either method alone. On the other hand, on the evidence presented, examination of pen faecal samples, whilst involving far less laboratory work, achieved similar results to those obtained by the examination of a far greater number of rectal swabs. In summary, 17 faecal sample examinations gave similar results to 104 rectal swab examinations. Both methods emphasized the need for repeated examinations before any assumption could be made that a pen was free from salmonella organisms.

Table 3. *Isolations of* Salmonella *from pig-pen faecal samples examined at fortnightly intervals from* 29 *March to* 12 *July* 1965

	Fortnightly period									
No. of pens examined	1 73	2 90	3 91	4 63	5 79	6 84	7 97	8 84	Totals 661	% positive
	Salmonella serotypes isolated									
S. typhimurium										
Type 1	4	1	2	1	1	0	0	0	.	.
Type 29	2	2	2	1	1	2	1	2	.	.
Type 30	0	0	1	1	2	2	0	0	.	.
Untyped	1	1	0	0	1	1	0	0	.	.
Totals	7	4	5	3	5	5	1	2	32	4·8
S. bredeney	11	6	15	6	6	7	9	9	69	10·4
S. durban	0	0	0	0	5	1	7	4	17	2·6
S. heidelberg	0	0	0	0	1	4	3	3	11	1·7
S. orion	2	0	0	0	0	0	0	0	2	0·3
Totals per fortnight	20	10	20	9	17	17	20	18	131	19·8
% salmonella-positive pens	27·4	11·0	22·0	14·3	21·5	20·2	20·6	21·4		

Since the epidemiological survey was to be based on the presence or absence of salmonellas from pens rather than individual animals, and in the light of the results of the comparison of methods reported above, it was decided to use pen-faecal sampling in this study. Each pen was sampled every 2 weeks, half the farm being sampled each week. The results were recorded on a ground plan of the farm, as were all the movements of pigs. Pigs were only moved for three reasons: when being moved to a new pen, during service and when moved for weekly weighing.

The farm consisted of six houses. These housed breeding sows and gilts, boars, farrowing sows and gilts, young boars on performance test, young weaned gilts and older gilts. During the investigations no males or females were retained after

they had produced one litter. The only animals to remain on the farm were vasectomized boars which occupied pens adjacent to the sow pens in the sow yard.

The bacteriological findings, over a 16-week period, are set out in Table 3. During the period five different sero-types were found to be present for part or the whole of the period, and for one of these, *Salmonella typhimurium*, three phage-types were identified. An average of 19·8% infected pens was recorded throughout the period. It must be emphasized that the infected-pen rate does not represent the salmonella pig carrier rate. In our experience the percentage of infected pens is always higher than the carrier rate as determined by individual rectal swab examinations. This is supported by an analysis of figures previously published (Table 4). In the present survey, an average of 19·8% infected pens would therefore most probably represent an individual salmonella carrier rate of the order of 8%.

Table 4. *The percentage of salmonella infection by individual pig examinations compared with the percentage of pens infected*

(Data from Heard *et al.* 1965).

	% salmonella infection	
Sampling date (1964)	Pigs infected (by rectal swabs)	Pens infected
6 July	8·3	25·3
27–30 July	7·2	26·0
14–17 September	24·2	56·0
29–31 December	15·9	37·0

ANALYSIS OF THE RESULTS

In order to correlate the presence of salmonellas in any particular pen with the movement of pigs within the farm, a number of arbitrary standards had to be laid down. It was decided to analyse within the scope of the 16-week period the history of all newly formed pens of pigs. This necessitated knowing the salmonella history of these pigs while in their previous pens. For inclusion in this survey these newly formed pens had to be continuously occupied by the same pigs for a period of not less than 4–6 weeks, that is, the time required to complete three fortnightly bacteriological examinations on pen faecal samples. Seventy eight pens met these requirements.

The decision to require a minimum of three bacteriological examinations was based on a number of observations. In this survey, 129 examinations were made on pens which proved to be infected with *Salmonella* but, of these, salmonellas were isolated from only 63 specimens. This suggests that we were successful in detecting salmonellas by our technique with only half the specimens. At least two examinations are therefore necessary to detect infected pens and the requirement of three examinations, we consider, gave reasonable coverage. Further, three fortnightly examinations were often the largest number which could be fitted in before animals became due for a routine move to another pen. We considered also that since in medical practice three negative specimens are often considered a

reasonable number before giving a human carrier clearance, this number was also reasonable in this present survey.

The findings of the seventy-eight pens and their subsequent pattern of salmonella excretion were followed. The pens were classified according to whether they were formed with pigs having a history of either a complete absence of salmonellas, or coming from pens from which salmonellas had been previously isolated. A further subdivision of the first class was made according to whether or not pens adjacent to the pen from which the pigs were taken had a history of salmonella infection. These three classes are set out in Table 5.

Table 5. *Epidemiological analysis of pen-sample results*

	History of pens from which new pens were formed			
	Pigs from salmonella −ve pens with adjacent pens −ve	Pigs from salmonella −ve pens with adjacent pens +ve	Pigs from salmonella +ve pens	Totals
No. of pens	11	26	41	78
Pens −ve after three examinations	11	18	28	57
Pens +ve during three examinations	0	8	13	21
Pens becoming +ve after three successive −ve results	2	2	4	8

DISCUSSION

The results can be adequately discussed only against the background of the strict hygiene measures taken on the farm. All attendants entering the farm were required to dress in newly laundered clothes and head covering and to put on gum boots used exclusively at the farm. Each house had its own attendant, and access to the house required the use of a disinfectant foot bath. The attendant entered each pen daily to brush it clean. Each pen was supplied with its own brush and care was taken to avoid carrying faeces from pen to pen within a house. Each week pigs from pens in the Early Rearing, Final Rearing and Performance Test Houses were weighed in the communal weigh pen. No precautions were taken between weighing each pen but full disinfection procedures were carried out between weighing pigs from each house. After a pen was emptied, before restocking, the walls and floor were thoroughly cleansed and disinfected and this was followed by a disinfectant/cement wash treatment of the walls. As a result of this procedure we have every confidence that no residual infection remained in the pens. During the period under survey, fifty-nine pens were cleaned and later restocked. Twenty-four of these were infected with *Salmonella* before and only six became subsequently infected after restocking and in each case an external source of infection was known. All houses save the sow and boar yards were

generally completely depopulated between pen occupation. The pens were of the usual commercial type, with concrete floors, cement rendered walls up to 3ft. 6 in. high, and galvanized iron fittings.

Although we have not isolated salmonellas from the pig food, there is considerable indirect evidence that the initial salmonella infection entered the farm in food. In July 1964 two salmonella serotypes were found; subsequently between July and December 1964 three other serotypes appeared (see Table 4). From December 1964 the food has been specially treated ensuring a minimum temperature of 145° F. in preparation followed by processing in small nuts which involves a further temperature treatment, and since this date no further salmonella serotypes have appeared. It would seem that for the last 12 months the outside source of infection has been controlled. Cross-infection on the farm must therefore be the result of activity within the farm. Birds are excluded from the houses and there is no evidence of rodent infestation. The main vehicles of cross-infection must be by direct faecal contact following pig movements, by pen mixing or service, by aerial spread or by the mechanical transfer of infection by the attendants.

In the light of these observations an interpretation of our results is possible.

In the first group of eleven pig pens, formed from non-infected pens with adjacent pens negative, all (100%) remained free from infection for a period of 4–6 weeks. Subsequently two became infected, but the source of these infections is not known.

In the second group, out of 26 pens, formed from previously non-infected pens, but with adjacent pens infected, 18 (69%) remained free from infection whilst 8 became positive. This would suggest that cross-infection between pens does occur possibly as a result of movements of farm attendants between pens, consecutive movement of pens for weighing or air-borne spread.

In the third group, of 41 pens formed from previously infected pens, 28 (68%) subsequently proved to be non-infected within the 4- to 6-week period. From these observations it may be concluded that in a high proportion of pens (in this case 68%) salmonella infection was self-limiting. Of these pens only 4 subsequently became positive during the duration of the trial, (i.e. up to 16 weeks).

From experience gained with this closed herd of pigs we are convinced that the major source of cross-infection is contact between healthy stock and infected faeces. This occurs when pens are mixed together, following pen-to-pen transfer of infection on the boots of farm attendants or following the movement of pens through a communal weighing area. This mode of cross-infection could also explain two infections which occurred following service of sows by infected boars.

By excluding an external source of infection it was possible to predict, with a fair degree of certainty, the position of pen infection simply by recording the movements of animals, and knowing their original bacteriological status.

As a result of the breeding programme on this farm it has not been possible to introduce all the control measures (especially the avoidance of excessive movement of pigs) which we are confident would speed up the reduction in the rate of cross-infection. Having controlled the external source of infection, the conditions necessary for salmonella control would seem to be: (1) the penning of pigs in

small groups with no contact with faeces of other pigs on the farm; (2) observing elementary hygiene measures when moving pigs from one house to another; (3) keeping pen-to-pen movement of both pigs and attendants to a minimum.

It seems reasonable to suggest that the methods of investigation on this farm provide a system for tracing enteric infection which might well be applied on a wider national and international scale, and should be of prime importance in the designing of farming systems.

SUMMARY

An epidemiological survey of salmonella infection in a closed herd of pigs is reported. The main cause of cross-infection resulted from the movement of animals, with subsequent mixing of infected and clean stock and exposure of non-infected animals to infected faeces. Pen-to-pen infection carried by farm attendants also proved important.

An extended survey is at present being conducted to gain further information on the processes of cross-infection.

We wish to acknowledge the practical help and advice given throughout this survey by the staff of the pig unit, by Professor Grunsell, Department of Veterinary Medicine, University of Bristol, and Professor Cooper and the technical staff of the Department of Bacteriology, University of Bristol. We are indebted to Dr E. S. Anderson for phage-typing the strains of *Salmonella typhimurium*.

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The infectivity assay of foot-and-mouth disease virus in pigs

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The ability to assay the infectivity of a virus preparation for a particular host is an important requisite in the study of that virus–host relationship. It can provide an estimate of the degree of adaptation or attenuation of a virus strain for the host and of resistance, either natural or acquired, possessed by an individual or a group of animals.

The method of titration of foot-and-mouth disease virus (FMDV) in cattle by inoculation of the tongue (Henderson, 1949) has formed the basis for many of the standard procedures used in FMDV research involving cattle. Comparatively little work, however, has been recorded on the development of suitable methods of titration of FMDV in pigs. Brooksby (1950) inoculated individual animals with single dilutions of virus intradermally in the tongue and snout in his studies of virus strains showing natural adaptation to swine. Graves & Cunliffe (1960) carried out some comparative work on the sensitivity of pigs to different routes of inoculation and concluded that the inoculation of the coronary band region of the foot was the most sensitive for the detection of virus. They developed a titration scheme using the simultaneous inoculation of several dilutions of virus into individual animals and showed that their results were reproducible within good statistical limits. Lucam, Dhennin, Dhennin & Fedida (1962) subsequently compared titration end-points using coronary-band and snout inoculation and confirmed the high sensitivity of the coronary-band region. Burrows (Communication to the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth-Disease, June 1963, summarized in F.A.O. document 15766/E) reported that the bulb of the heel of the foot was a convenient and highly sensitive site of inoculation for titration, innocuity testing and challenge of immunity procedures. Some details of the use of this method for these purposes have been recorded (Girard *et al.* 1963; Burrows, 1964*a*, *b*).

It is desirable, when proposing an assay method, to attempt to determine the precision of the method and also to compare the results with those given by established titration procedures. This paper presents the results of a number of comparative titrations of some FMDV strains in cattle, mice and pigs, with particular reference to the results obtained in pigs by the bulb of the heel inoculation method.

MATERIALS AND METHODS

Experimental animals

Details of the supply, housing and maintenance of cattle under experiment have been given by Henderson (1952). Similar details in relation to pigs have been recorded by Burrows (1964*a*).

Virus strains

Strains virulent for cattle

(*a*) A–119 Pirbright stock cattle strain used at the 26th cattle passage (*C* 26); (*b*) A–Kemron (Komarov & Goldsmit, 1958), *C* 2 (Pirbright); (*c*) O–Israel 1/63 World Reference Laboratory sample (WRLS), *C* 3; (*d*) C–997 British field sample (1953), *C* 6; (*e*) Sat 1–SA. 13/61 (WRLS), *C* 3; (*f*) SAT 1–Sudan 2/61 (WRLS), *C* 3; (*g*) SAT 1–Turkey 323/62 (WRLS), *C* 3; (*h*) SAT 3–SA. 57/59 (WRLS), C 5.

Seitz EK filtrates were prepared from 1/25 (w/v) suspensions of bovine tongue vesicle epithelium harvested 18–24 hr. after tongue inoculation. The suspensions were prepared in a mixture of equal volumes of M/25 phosphate buffer (pH 7·6) and Hartley's digest broth. Filtrates were held at 4° C. and usually titrated on the day of preparation. If titration was to be delayed for more than 24 hr., an equal volume of glycerol was added to the filtrate and the preparation stored at − 20° C. Survival under these conditions was satisfactory.

Strains modified for cattle

A number of modified strains derived by passage in adult mice (*mo* strains) or in embryonated eggs (*E* strains) were examined. Detailed results of titrations are recorded for the following strains: A–119 *mo*, A–Kemron *E*, O–M 11 *mo*, SAT 1–Israel *E* and SAT 3–SA. 57/59 *E*. The *mo* strains were used as glycerinated filtrates of infected mouse carcases (Skinner, 1960). The A–Kemron *E* and the SAT 1–Israel *E* strains were supplied by the Wellcome Laboratory, Pirbright, as concentrated tissue culture preparations. In addition, the results of bulb-of-the-heel titration of several other modified strains derived from the C–997 and SAT 3–SA. 57/59 virus strains were used to prepare Table 6.

An account of the behaviour in pigs of some of these modified strains has been recorded (Burrows, 1964*a*, *b*).

Virus assay

Cattle

Tongue inoculation (Henderson, 1949). Two cattle were inoculated intradermally in the tongue with four tenfold dilutions, using five sites on each tongue for each dilution.

Pigs

Bulb-of-the-heel inoculation (*heel*) (Burrows, 1964*a*). Four pigs were inoculated at two sites (inner and outer main digit) on each foot with a series of tenfold dilutions. This allowed the estimation of individual and group 50 % positive end-point dilutions. With certain groups of pigs the dilutions were distributed in a

factorial arrangement to allow additional calculation of group end-points based on particular feet.

Coronary-band inoculation (Graves & Cunliffe, 1960). Four pigs were inoculated with a series of tenfold dilutions following a pattern similar to that used for heel titration.

Tongue inoculation. Two or four pigs were inoculated intradermally in the tongue with four tenfold dilutions, using three sites on each tongue for each dilution.

Mice

Intraperitoneal inoculation (Skinner, 1951). Three to five litters of randomized 5- to 7-day-old 'P' strain mice were inoculated with a fivefold dilution series, using two subjects per litter for each dilution (Subak-Sharpe, 1961).

End-point determinations

The majority of cattle and all pigs were examined for lesions 18, 24, 30, 42 and 48 hr. after inoculation and daily thereafter. Mice were examined twice daily for 6 days. The 50% end-point dilution estimations were computed according to the method of Reed & Muench (1938) and were expressed as the number of 50% infective doses (ID 50) per ml. of the virus filtrate.

Table 1. *Time taken for vesicles to develop in relation to the concentration of virus inoculated into the heel*

	Number of ID 50* inoculated			
Virus	4	5–40	> 40	Secondary lesions
A–119	30†	28	23	42
A–Kemron	29	28	23	46
O–Israel 1/63	30	26	O.T.R.	41
C–997	26	26	24	42
SAT 1–Sudan 2/61	28	24	18	41
SAT 1–SA. 13/61	26	26	24	47
SAT 3–SA. 57/59	30	24	24	43

* Estimated from group end-point 30 hr. after inoculation.
† Mean time in hr. after inoculation.
O.T.R., Outside titration range.

RESULTS

The development of lesions following heel inoculation

The time of appearance of vesicles after heel inoculation was found to be dependent to some degree on the strain of virus and the concentration of virus inoculated and also on the individual animal. Table 1 records the mean times, in hours, required for vesicles to develop in relation to virus concentration for seven cattle strains of virus. Lesions were recorded once they had attained the diameter of 0·5 cm. The rate of vesicle growth was such that, with some strains of virus, they had involved some 2 cm.^{2} or more of the inoculation areas within 6 hr. of first appearance. Trials using two inoculation sites on each main digit were discontinued

because the rapid extension of a vesicle at one site frequently involved the second site before it had time to develop a visible reaction. Secondary lesions were recognizable in the majority of animals 40–48 hr. after inoculation and were first seen on the heel and coronary regions of the main digits and the coronary regions of the accessory digits. Vesicles on the tongue, lips and snout were usually apparent 72 hr. after inoculation and with certain strains of virus lesions were also observed on the skin covering the anterior surfaces of the limbs and on the teats and prepuce.

As a result of these observations, the final reading for end-point determinations was standardized at 30 hr. after inoculation of cattle strains of virus.

Lesions produced by virus strains modified for cattle were slower in development and limiting infective doses required 2 or 3 days to become apparent (Burrows, 1964*a*). Secondary lesions usually appeared on the 3rd or 4th day after inoculation.

Table 2. *Variation in experimental* 50 % *end-points observed in titration of cattle strains of virus using the 'heel' method*

Virus	Pig number 1	2	3	4	V
A–119	3·8	4·8	4·8	4·3	0·2291
A–119	3·3	5·3	5·8	4·3	1·2292
A–119	5·3	4·8	6·3	6·3	0·5624
A–Kemron	4·5	5·5	4·5	6·0	0·5624
O–Israel 1/63	5·5	5·0	4·5	6·5	0·7292
C–997	6·0	6·0	5·5	5·0	0·2291
SAT 1–Sudan 2/61	4·7	6·0	6·0	6·5	0·5993
SAT 1–SA. 13/61	4·8	6·8	5·8	6·3	0·7292
SAT 1–Turkey 323/62	4·6	5·6	5·6	4·6	0·3333
SAT 3–SA. 57/59	5·0	4·5	5·5	5·5	0·2291

Homogeneity of variance $\chi^2 = 5{\cdot}62$ (9), $> 0{\cdot}5$, $< 0{\cdot}9$; $\bar{V}$ 0·5432.

The variation of end-points determined by heel titration

An estimate of the total variation in the assay system can be obtained by calculating the variance of the individual 50 % end-points. The end-points recorded in ten titrations of cattle virus strains and eight titrations of modified virus strains are displayed in Tables 2 and 3. Differences in individual end-points within a titration group ranged from 0·5 to 2·5 $\log_{10}$ units with a mean of 1·34. The variances calculated for different titration groups ranged from 0·06 to 1·23. The results of Bartlett tests for homogeneity of the variances justify the calculation of average variances for (i) the cattle virus titration group (0·543), (ii) the modified strain virus groups (0·356) and (iii) the total data (0·460).

These variance values include components contributed by random sampling error and differences in susceptibility between digits and between animals. The experimental design does not allow assessment of the magnitude of these various components by an analysis of variance procedure. However, some general observations as to the major source of variation can be inferred from data collected from these and other experiments.

Differences between animals (breed and weight)

The pigs used in the majority of these experiments were commercial crossbreds (Wessex/Large White/Landrace) and ranged in weight from 45 to 70 lb. They were usually purchased in groups of at least ten and it is unlikely that all animals in a group were litter-mates. A single trial was carried out to see if there was any indication of extreme variation in the susceptibility of pigs of different breeds. The results of titration in purebred Large White (A), Landrace (B) and Wessex (C) showed no significant differences in end-points (Table 3, groups A–119 *moC*, A, B and C).

Table 3. *Variation in experimental* 50 % *end-points observed in titration of modified virus strains using the 'heel' method*

Virus	Pig number 1	2	3	4	V
A–119 *moC* A	2·0	3·5	3·5	2·5	0·5624
A–119 *moC* B	2·4	3·5	2·4	2·4	0·3058
A–119 *moC* C	3·0	2·0	4·0	3·5	0·7259
A–Kemron *E*	6·3	6·8	5·8	6·8	0·2291
SAT 1–Israel *E*	5·5	6·5	5·5	5·5	0·2500
SAT 3–SA. 57/59					
E 19	2·0	2·7	3·5	2·5	0·3891
E 30	2·5	2·5	2·5	2·0	0·0623
E 40	3·0	2·0	2·5	3·3	0·3266

Homogeneity of variance $\chi^2 = 5{\cdot}58$ (7), $> 0{\cdot}5$, $< 0{\cdot}9$; $\bar{V}$ 0·3564.

The weight of pigs has been shown to be of importance in the development of foot lesions following intramuscular inoculation of certain modified strains of virus (Burrows, 1964*a*). As pigs of slightly different weights were used in some titrations, this could have been a source of variation. However, titration of the O–M 11 *mo* virus strain in pigs of greatly different weight did not indicate that this factor was of importance with this particular virus strain; similar end-points were obtained with the two groups of animals (group 1–11 pigs, mean weight 69 lb., range 52–84 lb., mean end-point 3·7; group 2–16 pigs, mean weight 210 lb., range 140–284 lb., mean end-point 3·8).

Difference between digits

It is assumed that this error is probably not very great, i.e. that the eight main digits are of uniform susceptibility to virus, that identical inoculations are made at each of the sites and that similar volumes of inoculum are retained in each case. In respect of the latter two assumptions, the degree of wear and the thickness of the epithelium covering the bulb of the heel are variables of importance. These are fairly uniform within each pig but not necessarily uniform between different pigs. The validity of the assumption that the different feet are of equal susceptibility could be tested by using a large number of pigs and inoculating each foot with a small amount of virus; experiments of this nature were not attempted. Tests for

major differences in susceptibility were carried out by distributing virus dilutions in such a fasion as to allow the calculation of 50 % end-points on the responses of individual feet (group end-points) and also in each individual of the group. Table 4 gives the results of six titrations carried out in this manner. The variation in end-points estimated from the reaction of different feet (average variance 0·598) was of similar magnitude to that calculated from individual animal end-points (average variance 0·535) and no marked difference in sensitivity was noted.

Table 4. *Experimental* 50 % *end-points calculated in individual animals and on the feet of groups of animals*

	Individual animal end-points*					
				A–119 *moC*		
	SAT 1 SA. 13/61	SAT 1 Sudan 2/61	SAT 1 Israel *E*	A	B	C
Pig 1	4·6	4·7	5·5	2·0	2·4	3·0
2	6·8	6·0	6·5	3·5	3·5	2·0
3	5·8	6·0	5·5	3·5	2·4	4·0
4	6·3	6·5	5·5	2·5	2·4	3·5
Group $\bar{x}$	5·93	5·8	5·75	2·88	2·63	3·13
			$\bar{V}$, 0·535			
	Group end-points calculated on one foot of each animal					
Right fore	4·7	5·6	6·0	2·5	2·8	3·0
Left fore	5·5	4·7	5·5	3·0	1·8	4·5
Right hind	6·5	6·0	6·5	2·5	3·5	2·5
Left hind	5·5	7·0	5·0	3·3	3·6	2·5
Group $\bar{x}$	5·55	5·83	5·75	2·83	2·93	3·13
			$\bar{V}$, 0·598			
Group overall†	5·60	6·00	5·85	2·87	2·66	3·29

* These results also appear in Tables 2 and 3.
† Group end-point estimated in the standard way from the whole group.

Table 5. *Distribution of foot lesions on the main digits of pigs following intramuscular inoculation of seven modified strains of virus*

	Fore feet		Hind feet	
Site of lesion	Outer digit	Inner digit	Outer digit	Inner digit
Coronary-band area	60*	45	62	36
Bulb of the heel	54	47	58	38

* No of lesions found in fifty-four reactors.

Previous experience of the response of pigs to intramuscular inoculation of modified virus strains had indicated that the outer (IV) digit was more likely to develop secondary lesions than the inner digit (III). Table 5 details the distribution of foot lesions recorded on the main digits of fifty-four animals which developed lesions following intramuscular inoculation. The difference in the frequency of

occurrence of lesions on the outer and inner heels is significant at the 1% level (χ^2 6·8). A similar difference was also observed in the distribution of lesions involving the coronary band area.

The results of replicate inoculation of the heels of the titration animals were analysed in similar fashion (Table 6). No significant difference in the frequency of occurrence of lesions on the two heels could be shown in those animals used for titration of cattle strains of virus, but a slight but significant difference was observed in animals used for titration of virus strains modified for cattle.

Table 6. *Responses of outer and inner heels to replicate inoculation—titration animals*

Virus	No. of strains	No. of pigs	Outer heel	Inner heel	χ^2	P
Virulent for cattle	8	40	93/160*	83/160	1·26	0·5–0·1†
Modified for cattle	15	99	228/372	181/372	11·99	0·001

* No. of positive sites/no. of sites inoculated.
† Not significant.

Table 7. *The frequency of positive reactions to heel inoculation in relation to the concentration of virus inoculated*

	Virus strains virulent for cattle			Virus strains modified for cattle		
Approximate number of ID 50 inoculated	No. of sites	No. of positive sites		No. of sites	No. of positive sites	
		Observed	Expected		Observed	Expected
10	116	113	116	94	87	94
3	40	33	35	42	31	37
1	40	18	20	22	11	11
0·3	40	7	8	40	9	7
0·1	84	5	⩽ 8	58	6	⩽ 5
	320	χ^2	1·63	256	χ^2	2·26

Additional evidence for the assumption that the 'between digit' component is negligible was obtained from the titration animals by observing the frequency of reaction of sites inoculated with progressive dilutions of virus and testing this for agreement with expectation from a Poisson distribution (employing the conversion 1 ID 50 = 0·693 infectious units). The approximate number of ID 50 inoculated at each site was calculated for each animal from the end-point observed in that animal. Table 7 lists the data for both the cattle virus and the modified strain virus groups. Chi-square values were not significant but a larger value was determined for the modified strain group of animals and there was a trend towards a flatter dosage response curve.

Residual variation

This appears to be the major source of variation in the titration system. Interbreed differences in susceptibility were not marked and differences in susceptibility between digits were not demonstrably large. Thus, the main source of

variation would appear to be the result of other causes, i.e. the sum of the theoretical statistical minimum variance, differences in inoculum volume and differences in individual susceptibility. Clinical observation of the development and character of lesions following heel inoculation indicated that differences in individual susceptibility probably account for the major part of this residual variation.

The precision of end-points determined by heel titration

The average variance of 0·46 determined for the eighteen titrations gives a standard deviation of 0·68 for individual end-points. The 95 % confidence limits for a mean end-point can be determined for any number of animals, using the expression $(t_{17} \times \sqrt{0{\cdot}46})/\sqrt{n}$. Thus, the precision of an end-point based on four animals equals $\pm 0{\cdot}718$ and that based on nine animals $\pm 0{\cdot}479$.

Table 8. *Results of comparative titration of cattle strains of virus*

Virus	Mouse intra-peritoneal	Cattle tongue	Pig		
			Heel	Coronary	Tongue
A–119	7·5*	6·1	4·6	2·4	2·4
A–Kemron	6·5	5·8	5·2	1·3	–
O–Israel 1/63	7·2	6·7	5·3	3·0	–
C–997	7·0	5·6	5·6	2·7	3·9
SAT 1–SA. 13/61	7·9	7·2	5·6	–	–
SAT 1–Sudan 2/61	7·5	6·5	6·0	3·3	–
SAT 1–Turkey 323/62	7·8	6·6	5·1	2·6	4·1
SAT 3–SA. 57/59	7·9	6·4	4·9	4·0	4·0

* Log_{10} ID 50 per ml. of a 1/25 EK filtrate of bovine tongue epithelium.
–, Not tested.

Titration in pigs by other routes of inoculation

Coronary band inoculation

The development of primary lesions and the subsequent appearance of secondary lesions followed a pattern similar to that described for those following heel inoculation. It was found, however, that considerably more virus was required to produce visible lesions at the coronary-band site (Table 8). The variation in response of individual pigs was greater than that determined for heel inoculation. The average variance determined for eight titrations was 0·742 with 95 % confidence limits of 0·489–1·236 (the 95 % confidence limits for the average variance of end-points obtained by heel inoculation equalled 0·344–0·652). The standard deviation of the mean end-point determined in four animals ($\pm 0{\cdot}43$) was not substantially different from that of $\pm 0{\cdot}34$ recorded by Graves & Cunliffe (1960) in their original description of this titration procedure.

Tongue inoculation

Some difficulty was experienced in confining the inoculum to the thin epithelial layer and subsequent examination of the tongue for the presence of lesions could only be carried out satisfactorily with the pig under general anaesthesia. The results of four titrations are recorded in Table 8.

Primary lesions produced by tongue inoculation were inconspicuous, showed little tendency to extend and had usually healed within 48 hr. With some strains of virus and in certain pigs neither primary nor secondary lesions developed.

Comparative titration in cattle, pigs and mice

The results of comparative titrations of cattle strains of virus are given in Table 8. With all strains, mice gave slightly higher infectivity end-points than cattle (mean difference 1·05 $\log_{10}$, range 0·5–1·5). Titration in pigs by the heel route gave infectivity end-points approximately tenfold lower than those recorded for cattle (mean difference 1·07, range 0·0–1·6). Titration in pigs by the coronary-band route gave infectivity end-points several thousandfold lower than those found for cattle (mean difference 3·54, range 2·8–4·1). Tongue titration in pigs gave end-points similar to those determined by coronary-band inoculation.

The results of comparative titration of virus strains modified for cattle have already been recorded (Burrows, 1964*a*).

DISCUSSION

The observation that lesions appeared only on the bulbs of the heels of pigs after intramuscular inoculation of certain modified strains of virus led to the investigation of the susceptibility of the area to inoculation. Titrations comparing the sensitivity of the heel area with that of the coronary band region have shown the heel to be consistently more sensitive to those strains of FMDV which have been tested.

This higher sensitivity might be accounted for by the more efficient exposure of the basal layers of the epithelium to virus. The heel procedure consists essentially of making a needle track in the depths of the epithelium without entering the sub-epithelial region. Inoculation requires a considerable amount of pressure and the bulk of the inoculum (0·1 ml.) escapes when the needle is withdrawn owing to the dense and elastic nature of the heel epithelium. The amount of inoculum retained is thought to be fairly constant and is likely to be determined by the thickness of the needle and the length of the inoculation track. Syringes capable of accurate measurement of volumes less than 0·1 ml. were not sufficiently robust or suitable for the heel inoculation procedure. All infectivity estimates were calculated on the basis of a 0·1 ml. inoculum although it was recognized that the true infectivity of a virus preparation for the pig heel would be underestimated. A similar situation has been demonstrated in the calculation of infectivity estimates based on tongue inoculation of cattle (Hyslop & Skinner, 1964–see below).

In comparison, inoculation of the coronary band consists of an oblique penetration of the epithelium of the area and the delivery of the inoculum deep to the hoof wall. Little resistance is experienced to inoculation and experiments have shown that much of the inoculum is dispersed in the subcutaneous region underlying the coronary band and in the region between the hoof wall and the laminar matrix.

The susceptibility of pigs to the cattle strains of virus used was not much less than that of cattle. In six of the eight trials, uninoculated control pigs were housed

in contact with titration animals. In two trials (C–997 and O–Israel 1/63) some or all of these control animals did not develop clinical lesions. All these 'insusceptible' animals were later shown to have acquired a subclinical infection. Specific neutralizing antibodies were present 10 days after exposure and the animals did not develop secondary lesions when challenged in the heel with 100–1000 ID 50 (pig heel). Thus, failure to develop clinical lesions after a period of exposure to infection does not necessarily imply insusceptibility, an observation which has been noted on previous occasions at this Institute (Henderson & Brooksby, 1948; Burrows, 1964*b*) and by Nathans (1965).

Comparisons of infectivity titres in mice and cattle have usually been expressed in terms of end-point dilutions and usually these have been found to be similar for titration of cattle strains of virus (Skinner, Henderson & Brooksby, 1952; Heatley, Skinner & Subak-Sharpe, 1960). Conversion of these measurements to ID 50/ml. results in end-points approximately 0·5 $\log_{10}$ units higher being recorded by mice inoculated by the intraperitoneal route. The mean difference in end-points (ID 50/ml.) between mice and cattle determined in the present experiments has been somewhat greater; mice have given end-points approximately 1·0 $\log_{10}$ units higher than cattle. Four of the eight comparative titrations were carried out using aliquots from a master dilution series to obviate the minor errors inherent in preparing replicate series of dilutions up to 10^{-7}.

Hyslop & Skinner (1964) have speculated on the precise amount of the dose inoculated into the cattle tongue which contributes to the initiation of a local lesion. From preliminary observations they have indicated that the true infectivity for cattle of a virus suspension titrated in cattle by the standard method could well be 20-fold higher than that calculated on the inoculated dose per site of 0·1 ml. The results obtained in the series of titrations catalogued in Table 8 would support this view.

Essential requirements for the evaluation of the efficacy of FMD vaccines for pigs include the ability to titrate the challenge virus and a challenge procedure which will produce both primary and secondary lesions with regularity in the non-immune animal. If immunity is to be evaluated by a method akin to the Lucam K test (Lucam & Fedida, 1958), a titration technique which is capable of analysis by statistical methods is a prerequisite. Inoculation of the bulb of the heel fulfils all these requirements and has been used regularly during the past three years at this Institute for titration and for the challenge of animals vaccinated with both inactivated vaccines and modified virus strain vaccines.

SUMMARY

FMDV strains were titrated in pigs by inoculation of the bulb of the heel of the foot, the coronary band region of the foot, and the tongue. Heel inoculation gave end-points approximately 300-fold higher than did the other two methods.

Parallel titrations in cattle and mice gave infectivity estimates approximately 10-fold and 100-fold higher, respectively, than did heel titration of pigs.

The average variance of individual end-points determined for heel titration was

0·46 ($\log_{10}$ units) and that determined for coronary-band titration was 0·74. The sources of variations in end-points given by heel titrations are discussed.

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The mathematical analysis of concurrent epidemics of yaws and chickenpox

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INTRODUCTION

Mathematical analysis of factual epidemiological information is only slowly gaining recognition as a useful, and frequently preferable, method in the interpretation of epidemiological phenomena. In spite of a large amount of information on the epidemiology of yaws becoming available from large-scale eradication campaigns against yaws, much remains unclear. This is particularly true with respect to precise epidemiological parameters, such as the infection rate, which are vital for the planning of long-term surveillance of yaws in places where the prevalence of the disease has been brought down to a low level.

Periodic epidemic spread of endemic disease or long-term persistence of infectious diseases in populations in which otherwise the endemicity level would appear to be below the threshold level constitutes a puzzling aspect in epidemiology.

In this paper a mathematical analysis is employed to elucidate one epidemiological factor in yaws in a tropical area and to demonstrate the importance of a seemingly unrelated disease in changing the epidemiological pattern.

One of the authors (J.L.d.V.) investigated in 1957 an epidemic of yaws among children 10 years of age and younger† in a village of Netherlands New Guinea. The epidemic was of particular interest in that during it an epidemic of chickenpox broke out among the same population. Furthermore, it occurred a year and a half after a control survey during which total mass treatment (Hackett & Guthe, 1956; Report, 1960) had been applied in a WHO–UNICEF assisted yaws control campaign. Moreover, the spread of the disease was much faster than that normally found in yaws in Netherlands New Guinea (Kranendonk, 1958); there being a spectacular increase in the number of yaws cases in the month following the chickenpox epidemic. It was inferred that the chickenpox predisposed these children to become infected with yaws. This conjecture has some medical validity since:

(1) The incubation period of yaws is 20–30 days, usually assumed as 3 weeks

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† In holo-endemic areas, the percentage of infected children at age 5 is 95% (Kranendonk, 1958) and in general the susceptibles are exhausted at age 6 (Soetopo & Wasito, 1953).

(Turner & Hollander, 1957; Hackett, 1963), a time-gap consistent with the interval between the peaks of the chicken-pox and yaws epidemics;

(2) The spirochete causing yaws (*Treponema pertenue*) requires a portal of entry (Hackett, 1960) which might be provided by the skin eruptions caused by the chickenpox;

(3) The infection with chickenpox could cause increased susceptibility to yaws by stimulation of steroid production (Turner & Hollander, 1957).

This paper is concerned with advancing statistical and mathematical arguments relating to two specific aspects of these epidemics, namely:

(1) The children who had chickenpox were more likely to contract yaws in the month following the chicken-pox epidemic.

(2) The chicken-pox epidemic accelerated the yaws epidemic; that is, more cases of yaws were found than would otherwise have been the case.

The latter point is investigated using a mathematical model for a simple deterministic epidemic. A wider analysis relating to more general aspects of the epidemic is in preparation.

THE YAWS EPIDEMIC

Between August 1956 and February 1957 there was an epidemic of yaws among children 10 years of age and younger of Jongsu Besar in Netherlands New Guinea. In October of 1956 an epidemic of chickenpox occurred among the same population. The incidence of yaws by month was recorded retrospectively by one of the authors (J.L.d.V.) and is given in Table 1. In this table the children are also divided into two groups as to their previous history of yaws. This history is based on the information from accompanying parents and on the presence or absence of scars from infectious lesions, the latter being the arbiter when present. Absence of detectable scars and positive history is noted as positive, mainly because early infectious yaws does not always leave scars on healing.

A marked peak of the yaws epidemic is noted in November, the month following the chickenpox epidemic. Later the epidemic gradually builds up in an epidemio-

Table 1. *Course of yaws epidemic according to yaws history*

	New yaws cases		
Month	Negative history of yaws	Positive history of yaws	Total
Aug. 1956	1	0	1
Sept. 1956	1	0	1
Oct. 1956	0	0	0
Nov. 1956	11	2	13
Dec. 1956	2	0	2
Jan. 1957	3	1	4
Feb. 1957	8	2	10
Unknown but after Nov. '56	3	2	5
Total cases	29	7	36
No. uninfected	26	41	67
Totals	55	48	103

logical pattern typical for yaws to a second peak in February. At this point mass treatment with long-acting penicillin (PAM) brought an end to the epidemic.

Table 2 records the monthly incidence of yaws among children 10 years and younger by the chickenpox infection status. It is noted that 12 of the 13 cases of yaws in November had chickenpox in October while of the 14 cases in January and February only 3 were of the chickenpox group.

Table 2. *Course of yaws epidemic in children aged* 10 *years or less according to chickenpox history*

Chickenpox status	Total no. of children	Yaws cases in: Aug.–Oct.	Nov.	Dec.	Jan.–Feb.	Unknown	Total Yaws
Yes	50	2	12	1	3	1	19
No	49	0	1	1	11	1	14
Unknown	4	—	—	—	—	3	3
Total	103	2	13	2	14	5	36

Except for five individuals among the total population of the village, all the chickenpox cases occurred in October. The five exceptional cases involved only two children who were 10 years of age or younger. One of these had chickenpox in September and already had a positive history of yaws. The second had chickenpox in November and had a negative history of yaws. None of the five contracted yaws during 1956 or 1957.

PRELIMINARY STATISTICAL ANALYSES

We shall consider first whether the chickenpox group had a significantly higher incidence of yaws in November than did those without chickenpox. There were 48 susceptibles among the former group in November of which 12 contracted yaws, for an incidence rate of 25% (see Table 2). On the other hand only 1 of 49 susceptibles in the non-chickenpox group contracted yaws in November for a rate of less than 2%. This difference is highly significant ($\chi_1^2 = 11{\cdot}02$, $P < 0{\cdot}001$). Turning to the incidences in January and February, we find 8·6% incidence of yaws among the chickenpox group (3 of the 35 remaining susceptibles), but 23·4% incidence of yaws among the non-chickenpox group. This difference approaches significance ($\chi_1^2 = 3{\cdot}12$, $P = 0{\cdot}08$). These results indicate a strong short-term association between chickenpox and yaws. These statistical tests should be adjusted to take into account the third variable of classification, the yaws history. Since the history status turns out to be distributed about proportionately between the other two classes, these conclusions are sustained by more sophisticated statistical tests which take into account the yaws history.

We turn to Table 1 to consider whether the distribution of cases could be described by a random process rather than the more mathematical model which we shall later consider. The simplest random model would be a Poisson process, for which the mean and variance are the same. We find for the distribution of cases by month

(excluding the unknown category) that the sample mean $\bar{x} = 31/7 = 4{\cdot}43$, while the sample variance $s^2 = 25{\cdot}62$. The Lexis ratio is $s^2/\bar{x} = 5{\cdot}78$, a highly significant departure from randomness ($\chi^2_6 = 6(s^2/\bar{x}) = 34{\cdot}71$, $P < 0{\cdot}001$). Since we are concerned particularly with the month of November, we shall compare the incidence of yaws in that month with the preceding months. The rate of cases per month for August to October inclusive is 2/3, while for November it is 13. Assuming Poisson variation for these months, we may test this difference by an F statistic (Cox, 1953) for which we find $F(5, 27) = 16{\cdot}2$, $P < 0{\cdot}001$. Taken together these results indicate these data cannot be adequately described by a Poisson process and that the month of November is exceptional when compared to the previous course of the epidemic.

REVIEW OF THE MATHEMATICAL MODEL

Bailey (1957) discusses a deterministic model for a simple epidemic with no removal which is 'approximately applicable to the sort of situation where (*a*) the disease is highly infectious but not sufficiently serious for cases to be withdrawn by death or isolation, and (*b*) no infective becomes clear of infection during the main part of the epidemic'. Yaws qualifies as a suitable disease for this model.

Let $X(t)$ be the number of susceptibles in the population at time t and let $Y(t)$ be the corresponding number of infectives. The basic model assumes the incidence is proportional to the product of the number of infectives and the number of susceptibles (i.e. the number of possible pairwise contacts). This is the 'homogeneous mixing' postulate and is mathematically written

$$\frac{dY(t)}{dt} = \beta\, Y(t)\, X(t), \tag{1}$$

where β is a constant infection rate. We solve equation (1) subject to the boundary conditions, $Y(0) = 1$ and $X(0) = n$; that is, we assume initially one infective and n susceptibles in a population of size $n+1$, which remains constant during the course of the epidemic. The resulting solution in terms of $Y(t)$ is the logistic (or autocatalytic) curve,

$$Y(t) = \frac{n+1}{1+ne^{-(n+1)\beta t}}. \tag{2}$$

This equation may also be written in the form*

$$L(t) = \frac{1}{n+1} \ln\left[\frac{nY(t)}{n+1-Y(t)}\right] = \beta t. \tag{3}$$

$L(t)$ will be called the logit at time t. We note that the logit graphs linearly against time and thus provides a simple test of the model. It is also of interest to note that $dY(t)/dt$, the epidemic curve, is a bell-shaped curve with maximum at

$$t_{\max.} = (\ln n)/[\beta(n+1)], \tag{4}$$

the time at which the incidence of the disease is the greatest.

* ln denotes natural logarithm.

THE NUMBER OF SUSCEPTIBLES

In order to apply this model we must define a population size for the number of susceptibles. This size is not obvious and ascertainment of it is a difficulty associated with fitting other epidemic models (cf. Abbey, 1952). We cannot limit this population to those with a negative history of yaws since, in fact, seven cases also occurred in the positive history group. We consider two approaches to determining an 'effective population size'.

Assume that the positive history group can be divided into subpopulations of two discrete types. One subpopulation is wholly resistant to yaws and this does not contribute to the population of susceptibles. The second subpopulation is of the same degree of susceptibility as the negative history group in determining the effective population size. We estimate this size of this second subpopulation by assuming that the proportion having yaws in this subpopulation should be the same as that in the negative history group, that is, $\frac{29}{55}$ or 52·7%. All seven yaws cases would have occurred among the subpopulation and so we can solve back and find its size, namely $7 \times \frac{55}{29}$ or about 13. Thus, on the basis of the assumption, the effective population of susceptibles will be estimated at $55+13 = 68$.

A second argument assumes that two different infection rates β_1 and β_2 operate among the negative and positive history groups respectively. If we assume that $\beta_1/\beta_2 = \rho$ is large (compared to one) and that the number of cases among the negative group is small, it is possible to describe the epidemic by a modified form of (1) wherein $\beta_1 = \beta$ and the effective population size is

$$n+1 = n_1+\frac{n_2}{\rho}+1, \tag{5}$$

where n_1+1 and n_2 are the sizes of the negative and positive history groups respectively. The ratio ρ may be estimated by

$$\hat{\rho} = \frac{\ln(x_1/n_1)}{\ln(x_2/n_2)}, \tag{6}$$

where x_1 and x_2 are the number of remaining susceptibles in the two populations at the end of the epidemic.* For this example we find from (6) that

$$\hat{\rho} = \frac{\ln 54-\ln 26}{\ln 48-\ln 41} = 4{\cdot}636$$

and the effective population size estimated from (5) to be

$$n+1 = 55+\frac{48}{4{\cdot}636} \sim 65.$$

As can be shown to be the case under certain conditions, these two arguments lead to approximately the same results. We shall arbitrarily use sixty-eight as the effective population size although in the following analyses the qualitative results are not substantially affected by which of the two numbers is actually used.

* The mathematical details of this argument have been derived by one of the authors (J.J.G.) and will be published elsewhere.

APPLICATION OF THE MODEL

In order to apply the model we must distribute the 5 unknown cases in Table 1 among the months after November. We arbitrarily assigned 1 of these to December, 2 to January and 2 to February, arriving at the totals given in Table 3. We shall see that the critical part of the analysis is invariant with respect to the specific allocation of these 5 cases among these 3 months. In Table 3 we have denoted the end of August by $t = 0$, the end of September by $t = 1$, etc. The last column of Table 3 gives monthly estimates of the infection rate given by taking differences among the adjacent logits, that is, $L(t) - L(t-1) = \hat{\beta}_t$, a result following from (3). We note that the infection rate for November is 0·03286 or over three times that

Table 3. *The adjusted data and the analysis using the model*

Month	t	New yaws cases (adj. totals)	$Y(t)$	$n+1-Y(t)$	$L(t)$	$\hat{\beta}_t$
Aug. 1956	0	1	1	67	0·00000	—
Sept. 1956	1	1	2	66	0·01041	0·01041
Oct. 1956	2	0	2	66	0·01041	0·00000
Nov. 1956	3	13	15	53	0·04327	0·03286
Dec. 1956	4	3	18	50	0·04681	0·00354
Jan. 1957	5	6	24	44	0·05292	0·00611
Feb. 1957	6	12	36	32	0·06357	0·01065

for any other month. The arithmetic mean of the rates excluding November is 0·00614, while for the first 2 months it is 0·00520 and for the last 3 months it is 0·00677. It is of interest to note that the mean infection rates before and after the chickenpox epidemic are comparable. We also note that the mean infection rate between times t and $t+a$ may be alternatively calculated from the formula $\bar{\beta} = [L(t+a) - L(t)]/a$. Thus the mean infection rate for the months following November is invariant with respect to the assignment of the unknown cases among these months.

We now consider the projection of the epidemic from conditions prevailing before the chickenpox epidemic apparently accelerated the yaws epidemic. We substitute the mean infection rate for the first 2 months (0·00520) in (2) and find

$$Y(t) = \frac{68}{1 + 67e^{-0\cdot354t}}. \tag{7}$$

The theoretical and observed epidemics are graphed in Fig. 1, this theoretical curve being the solid curve. It is noted that the projected epidemic would result in a total of about eight cases by the end of February, while in fact thirty-six were observed. These twenty-eight may be regarded as the excess due to the chickenpox epidemic. We may also use (4) to find the peak of the projected epidemic,

$$t_{\max.} = \frac{\ln 67}{68(0\cdot00520)} = 11\cdot9,$$

or about the end of August 1957. The epidemic was actually ended in March 1957 by the intervention of treatment. Moreover, August 1957 would have produced

only eight new cases, while in fact peaks of thirteen and twelve cases were reached in November 1956 and February 1957 respectively.

We may also project the equation for the model from conditions prevailing at the end of November, after which time the effects of the chickenpox epidemic presumably no longer affected the course of the yaws epidemic. We solve (1) subject to the boundary conditions $Y(3) = 15$, $X(3) = 53$ and find

$$Y(t) = \frac{68}{1 + (\frac{53}{15})e^{-68\beta(t-3)}}. \tag{8}$$

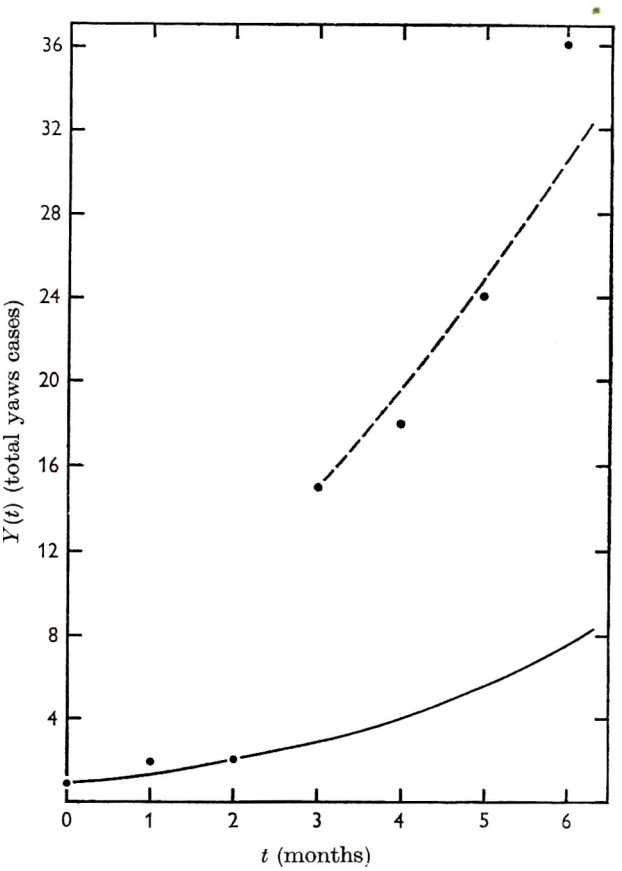

Fig. 1. The observed and projected courses of the yaws epidemic.
—, Projected from $t = 2$ (equation 7). - - -, Projected from $t = 3$ (equation 8).

We let β in (8) be the mean calculated from the first 2 months (0·00520) and find the theoretical curve given by the broken line in Fig. 1. We see that this curve approximately follows the actual course of the epidemic for this period. This reflects the fact that the average infection rates before and after November are roughly comparable.

The model as represented by (1) assumes that the population in question is subject to homogeneous mixing. We count all the possible contacts between infectives and susceptibles and make the incidence proportional to this number. This essentially weights all pairwise relationships between the individuals equally.

Actually we would expect the individuals in the same household to have more contact than those, for example, living on opposite ends of the village. The first two cases in the epidemic of yaws illustrate the case. The second case was a twin sibling of the first case. The next thirteen cases in November were outside this household. Thus the observed course of the epidemic might then be used as an argument against the assumption of homogeneous mixing.

Let us consider this more carefully. The infection rate between twins would probably be the maximum of any situation. The rate between households (October and November) should be smaller, but in fact we observe a much larger rate for November, the month after the chickenpox epidemic. This would argue even more forcefully for the proposed hypothesis.

Because of the small numbers of cases in the first few months, the projections based on the model are not too accurate. One of the referees has suggested that the numbers of cases in the months of November to February inclusive might be considered to arise from a long, rather flat peak. Testing for homogeneity the number of cases in these months by using the Lexis ratio test on adjusted data of Table 3 (13, 3, 6, 12) we find $\chi_3^2 = 8{\cdot}12$, $P < 0{\cdot}05$. However, an arbitrary assignment of the unknown cases could equally well have led to the pattern of cases 13, 5, 6, 10. Then $\chi_3^2 = 4{\cdot}82$, $P = 0{\cdot}18$. In this case the contrast of the months of November and February versus December and January leads to

$$\chi_1^2 = \frac{[(13+10)-(5+6)]^2}{4(8{\cdot}5)} = 4{\cdot}24.$$

Thus the contrast between the two apparent peaks and the intervening months accounts for a very large part of the variation observed. The P value associated with the test of this contrast is 0·04, although it must be noted that this contrast, like the arbitrary assignment, was chosen after the fact.

All these results seem to point to the chickenpox epidemic as a factor in the acceleration of the yaws epidemic.

SUMMARY

The relationship between chickenpox and yaws epidemics occurring among children in a village in Netherlands New Guinea is analysed using the mathematical model for a simple deterministic epidemic. It is shown that the yaws epidemic accelerated significantly in the month following the chickenpox epidemic, but that it reverted to its previous rate in the succeeding months. The number of yaws cases attributable to the influence of the chickenpox is estimated from the projected course of the yaws epidemic. It is statistically verified that those children contracting chickenpox were more likely to become yaws cases in the subsequent month.

These results point to the danger of yaws's spread being much more rapid among a population which has recently been subject to an epidemic of chickenpox.

We are grateful to Prof. Philip Sartwell and a referee for useful suggestions.

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The distribution of complement-fixing antibody and growth-inhibiting antibody to *Mycoplasma hominis*

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Mycoplasma hominis is a common inhabitant of the human genital tract. This organism can be isolated frequently from patients with genital infections and from those with no evidence of such infection (Harkness, 1950; Randall, Stein & Ayres, 1950). It is likely that *M. hominis* is a commensal and in general may be isolated more frequently from women than men (Nicol & Edward, 1953). The organism may also be a potential pathogen and the cause of genital and puerperal infections accompanied by the appearance of complement-fixing antibody in the serum of the patient (Stokes, 1959; Lemcke & Csonka, 1962). Mufson *et al.* (1965) have shown that *M. hominis* is capable of producing exudative pharyngitis in experimentally infected volunteers.

The growth of mycoplasms can be inhibited by specific antibody and Taylor-Robinson, Purcell, Wong & Chanock (1966) have developed a sensitive metabolic inhibition test which allows the measurement of growth-inhibiting antibody in human sera. An investigation was made into the occurrence and distribution of growth-inhibiting antibody to *M. hominis*, compared with the distribution of complement-fixing antibody in the same sera.

MATERIALS AND METHODS

Sera

The material used for this study was the sera received with 3200 consecutive requests sent to the Central Serology Laboratory, Manchester, between 1 and 9 February 1966.

Procedure

The sera were inactivated at 56° C. for 30 min. Using a Multiple Pipetting and Diluting Machine,* a 1/5 dilution of each serum was prepared and 0·1 ml. volumes of each dilution distributed in two series of MRC plastic trays. One series of trays was used for the screen complement-fixation test and the other series for the screen growth-inhibition test. The sera were stored at −20° C. until testing was completed. Trays containing doubling dilutions for the titration of sera giving positive results at 1/5 dilution were prepared in a similar manner.

* Shandon Scientific Company Ltd.

Media

The basic medium for growing *M. hominis* consisted of 7 parts Difco PPLO broth, 2 parts unheated human serum, 1 part boiled blood extract, 1/2000 thallium acetate and 1000 units penicillin/ml. For the inhibition tests the basic medium was used but with added arginine (1 %), phenol red (0·002 %), and fresh unheated guinea-pig serum (7 %), with the final pH adjusted to 7·0.

Growth-inhibition test

The test was performed in the manner of Taylor-Robinson, Purcell, Wong & Chanock (1966) but modified by replacing the glucose in the medium with arginine (Taylor-Robinson, Purcell & Chanock, 1966). The metabolism of arginine by the mycoplasms produces an increase in the pH of the medium resulting in a colour change. For convenience the test was performed in MRC plastic trays using 0·1 ml. as a unit volume. Preliminary chessboard titrations with a rabbit antiserum and different doses of organisms, prepared from 48 hr. broth cultures of *M. hominis*, were performed. The challenge dose of organisms chosen for the single row test was 10^5 colony-forming units per ml.; this allowed the results to be read after 30 hr. incubation at 34° C. The test in the final form consisted of adding 0·3 ml. of a mixture of basic medium, indicator, arginine, guinea-pig serum and organisms to 0·1 ml. of a dilution of patient's serum. All sera were tested at 1/5 dilution; any sera showing inhibition of colour change were later titrated. The tests were read when the end-point of the positive control serum was readable at its known titre (1/6000) and in this way the sensitivity of each batch of tests was kept constant. On further incubation the organisms tended to 'grow through' in the presence of small amounts of antibody. During incubation the plastic plates were covered with celluloid covers to avoid evaporation losses. The colour change was read against a white background with overhead fluorescent illumination.

Complement-fixation test

The complement-fixing antigen and immune rabbit serum were prepared by the method of Card (1959). Veronal buffer was used for the preparation of dilutions and reagents. The haemolytic system contained 2½ % sheep red cells sensitized with 5 M.H.D. of rabbit anti-sheep haemolysin.

Preliminary experiments confirmed that the test was most sensitive when incubated overnight at 4° C. The optimal working dilution of the antigen was estimated by a chessboard titration against immune rabbit serum and using minimum haemolytic doses of complement estimated by incubating the antigen and complement dilutions with negative serum under the normal test conditions of time and temperature. The complement dose for the test proper was estimated in the same way. A single pool of guinea-pig serum preserved by Richardson's method was used throughout the experiment at a constant dilution. All reagents were added to the prepared trays of dilutions with a Donaldson's dropper delivering 0·1 ml. volumes. The trays were shaken 15 min. after addition of sensitized cells and the test read after a further 45 min. at 37° C.

Analysis of results

The routine reporting at the Central Serology Laboratory utilizes a punch-card installation.* Analysis of cards produced during the period of the study permitted the analysis of the patients by age, sex and type of department attended. This information, together with the results of the two tests, was punched on a further card for each patient giving a positive reaction with one or both tests. These cards were then sorted into the required groups and totalled or reproduced as required.

RESULTS

Homologous rabbit serum

In a preliminary experiment various dilutions of *M. hominis* suspension were titrated against the homologous rabbit serum by the growth-inhibition technique. The highest dilution of serum that inhibited the colour change was 1/6000. The complement-fixation titre of this serum, with the batch of antigen used for this investigation, was also 1/6000. Serum was again collected from the rabbit 4 months after the post-immunization sample and re-examined. The complement-fixation titre had fallen to 1/400 whereas no growth-inhibiting property was detectable.

Human sera

A total of 3163 sera were tested by both techniques: in 13·5 % the complement-fixation test was positive and in 4·2 % the growth-inhibition test was positive. Six sera were found with a positive growth-inhibition test and a negative complement-fixation test. When these were divided on the basis of sex, 2265 sera were from female patients and 16·2 % contained complement-fixing antibody and 4·7 % growth-inhibiting antibody, while of sera from 560 male patients, 10 % were positive in the complement-fixation test and 4·6 % positive by the growth-inhibition technique. The results obtained with sera from each sex were then analysed separately in respect of age and titre of antibody and divided into various patient groups. In the remaining 338 sera, the sex or origin of the patient was not known and these results were not analysed further.

Female patients

The percentage distribution of complement-fixing antibody is shown in Table 1*a*. In the 0 to 5 year age group, antibody was present in one baby under 3 months old and was probably of maternal origin. One patient (who was pregnant) under the age of 15 also had antibody. With these exceptions the incidence of antibody increases through the sexually active years, the increase continues during the decade after the menopause and then the incidence declines. Assuming that the higher titres of 1/40–1/80 are likely to indicate more recent or severe infection there is the same increase in incidence of these titres up to the age of 55.

The percentage distribution of growth-inhibiting antibody is shown in Table 1*b*. Again there is a steady increase in incidence with age but with a sharper increase

* International Computors and Tabulators Ltd.

Table 1*a*. *Complement-fixing antibody for* Mycoplasma hominis *type I in* 2265 *female patients*

Age (years)	Complement-fixation titre 80	40	20	10	5	Tr.	(+)	Total per-centage	No. exam-ined
> 65	.	.	.	5·3*	.	.	.	5·3	57
55–65	.	.	6·2	4·1	.	2·0	.	12·4	48
45–55	2·4	9·5	2·4	4·8	.	4·8	2·4	26·2	42
35–45	3·3	3·9	6·1	4·4	2·8	1·1	0·5	22·1	181
25–35	1·8	3·2	4·0	4·3	0·5	1·3	.	15·2	618
15–25	1·9	3·4	3·3	2·1	1·5	1·7	0·6	14·4	953
5–15	.	.	.	10	.	.	.	10	10
0–5	.	.	.	.	.	.	2·7	2·7	37
Unspeci-fied	1·9	3·1	6·3	4·7	2·2	0·9	0·3	19·4	319

* Percentage positive.
(+), Positive result at 1/5 dilution but insufficient serum for titration.
Tr., Weak fixation at 1/5 dilution.

Table 1*b*. *Growth-inhibiting antibody for* Mycoplasma hominis *type I in* 2265 *female patients*

Age (years)	Growth-inhibiting titre 80	40	20	10	5	Tr.	(+)	Total per-centage	No. exam-ined
> 65	.	.	.	.	.	.	.	.	57
55–65	.	.	.	4·1*	.	2·0	.	6·1	48
45–55	.	.	2·4	4·8	4·8	4·8	.	16·7	42
35–45	.	0·5	0·5	3·9	1·6	.	.	6·6	181
25–35	.	0·3	1·0	1·0	1·1	0·5	0·2	4·1	618
15–25	0·2	0·1	0·5	1·2	1·3	0·6	0·3	4·2	953
5–15	.	10	.	.	.	.	.	10	10
0–5	.	.	.	.	.	.	2·7	2·7	37
Unspeci-fied	.	0·9	0·6	.	2·5	0·3	.	4·4	319

* Percentage positive.
(+), Positive result at 1/5 dilution but insufficient serum for titration.
Tr., Weak neutralization at 1/5 dilution.

Table 2. *General hospital patients*

Age (years)	Female: Total no. examined	Female: Percentage positive by CFT	Female: Percentage positive by GIT	Male: Total no. examined	Male: Percentage positive by CFT	Male: Percentage positive by GIT
> 65	56	3·6	0	54	9·3	7·4
45–65	80	16·3	10·0	120	5·0	0·8
25–45	92	15·2	4·3	75	6·7	5·3
15–25	33	15·1	3·0	27	0	0
5–15	7	0	0	3	0	0
Unspecified	44	13·6	2·3	64	6·3	3·1
Total all ages	312	12·8	4·5	343	5·8	3·2

in the post-menopausal decade. The titres of growth-inhibiting antibody were generally lower than those obtained in the complement-fixation test. In female general hospital patients (Table 2) the incidence of antibody rises with age to a

Table 3. *Ante-natal patients*

Age (years)	Total no. examined	Percentage positive by CFT	Percentage positive by GIT
35–45	133	21·8	6·8
25–35	548	14·1	3·5
15–25	865	12·3	3·5
Unspecified	274	17·5	4·0
Total all ages	1820	14·4	3·8

Table 4. *V.D. clinic patients*

	Female			Male		
Age (years)	Total number examined	Percentage positive by CFT	Percentage positive by GIT	Total number examined	Percentage positive by CFT	Percentage positive by GIT
> 65	1*	+	−	1*	+	+
45–65	8	37·5	25·0	23	13·0	8·7
25–45	18	44·5	11·1	146	10·3	2·1
15–25	38	23·1	7·7	76	7·9	3·9
5–15	1*	+	+	0	0	0
Total all ages	66	33·3	12·1	246	10·2	3·7

* Positive result from the single patient in these groups.

Table 5. *Female inmates of H.M. prisons*

Age (years)	Total no. examined	Percentage positive by CFT	Percentage positive by GIT
25–45	10	30·0	30·0
15–25	19	26·3	26·3
Total all ages	29	27·6	27·6

Table 6. *Female patients of general practitioners*

Age (years)	Total number examined	Percentage positive by CFT	Percentage positive by GIT
25–45	31	9·7	3·2
15–25	32	12·5	3·1
Unspecified	40	17·5	7·5
Total all ages	103	13·6	4·9

maximum in the 45–65 age group and then declines. In antenatal patients (Table 3) the incidence of antibody also increases with age and complement-fixing antibody is approximately three times as common as growth-inhibiting antibody at all

ages. Female patients attending venereal disease clinics (Table 4) have a much higher incidence of antibody than the previous groups. The maximum incidence is in the 25–45 age group although the numbers examined in each category are small. A small group of female inmates of H.M. prisons (Table 5) showed a similar high incidence of antibody and in this group the results from both tests were the same and showed no significant age differential. The distribution of antibody amongst female patients of general practitioners, shown in Table 6, was very similar to that in general hospital patients.

Table 7. *All patient groups, males and females*

	Female			Male		
		Percentage positive by			Precentage positive by	
Age (years)	Total no. examined	CFT	GIT	Total no. examined	CFT	GIT
> 65	57	5·3	0	56	10·7	5·4
55–65	48	12·4	6·1	76	6·6	2·6
45–55	42	26·2	16·7	74	5·4	1·4
35–45	181	22·1	6·6	94	3·4	1·1
25–35	618	15·2	4·1	142	12·7	4·2
15–25	953	14·4	4·2	104	6·7	1·9
5–15	10	10·0	10·0	7	0	0
0–5	37	2·7	2·7	21	4·8	4·8
Unspecified	319	19·4	4·4	76	7·9	3·9
Total all ages	2265	16·2	4·7	560	10·0	4·6

Male patients

In general complement-fixing antibodies to *M. hominis* are less common in men than women, although the overall incidence of growth-inhibiting antibodies is about the same. The age distribution of antibodies differs between the sexes (Table 7). In men, as with the women, there is a steady increase in incidence with age. The higher incidence in the 25–35 year age group in this series is due to the disproportionately large number of V.D. clinic patients in this group. In men we find that the incidence of antibody continues to increase in the over-65 group whereas in women of this age antibody is less common. Male patients of the general hospital group (Table 3) show this difference particularly and a direct comparison is possible with the similar female group. Male V.D. clinic patients (Table 4) have a higher than average incidence of antibody amongst the males but much lower than the comparable female group.

Children

Forty-one specimens of serum were from children under the age of five. Two children, one male and one female, had both growth-inhibiting and complement-fixing antibody, both were less than three months old and it was assumed that these antibodies were of maternal origin.

DISCUSSION

In the population examined, growth-inhibiting antibody to *M. hominis* was rarely found in the absence of complement-fixing antibody and was less common. With a few exceptions the complement-fixation titres were higher than the growth-inhibition titres. The growth-inhibition test may be less sensitive than the complement-fixation test, although the converse has been shown to hold for other *Mycoplasma* spp. by Taylor-Robinson, Purcell, Wong & Chanock (1966). We think that growth-inhibiting antibody probably appears in smaller amounts only in the more prolonged or severe infections, and disappears more quickly than complement-fixing antibody. That this may be so is suggested by the behaviour of these antibodies in the hyperimmune rabbit. Personal observation of a small number of patients with puerperal *M. hominis* infections also supports this hypothesis.

Little evidence has been accumulated about the frequency of antibody to *M. hominis* in the general population. Studies that have been made have usually been on a small number of people and often with no reference to age or sex. Lemcke & Csonka (1962) found antibody in 4% of 109 female blood donors and compared this with the antibody incidence of 50% amongst 51 patients with salpingitis. Shepard (1954) has pointed out that, for comparison, groups need to be matched for age, sex, social class and also sexual promiscuity. This is clearly difficult but in this study we have taken a comparatively very large sample and picked out various easily defined groups to see what differences there are.

Antibody to *M. hominis* is uncommon below the age of 15 years, obvious exceptions being in very young babies with maternal antibody and in an unusually promiscuous female patient. After this age, the presence of antibody becomes more frequent with increase in age, although important differences are to be seen between the sexes. In women, apart from a generally higher incidence of complement-fixing antibody, we find that antibody reaches a peak in the post-menopausal decade, and is uncommon after the age of 65. This is in contrast to the men where the incidence of antibody rises sharply over the age of 65. This difference is possibly due to the genito-urinary troubles that beset men at this age and *M. hominis* may play a part in prostatic inflammation. Why the post-menopausal decade in women should be the period of highest incidence of antibody also requires explanation. It is possible that as the acidity of the vagina becomes less, this allows *M. hominis* to proliferate.

The incidence of growth-inhibiting antibody in men and women is about the same (4·6–4·7%) but complement-fixing antibody is commoner in women (16·2%, compared with 10% in men). This may mean that women tend to suffer from trivial infections that fail to generate neutralizing antibody, whereas in men, when *M. hominis* infection does occur, both neutralizing and complement-fixing antibodies are likely to appear.

The only other recent survey of antibody to *M. hominis*, of any size, was reported by Taylor-Robinson *et al.* (1965). These workers examined a total of 256 sera from patients of all ages using an indirect haemagglutination technique which was much more sensitive than the complement-fixation test in their hands. These

patients, who were not divided into sexes, showed a general increase in the incidence of antibody with age, reaching a maximum in the 40 to 49-year group. They were able to detect some antibody in a few children between 5 and 14 years of age.

The age incidence of complement-fixing antibody to *M. pneumoniae*, which is an accepted respiratory pathogen, is not the same as we find with *M. hominis*. Andrews (1965), who also examined sera collected in N.W. England, and Grayston, Alexander, Kenny & Clarke (1965), working in America, have shown that antibody to *M. pneumoniae* is present in children and reaches a maximum incidence in early adult life. This distribution is quite unlike that of *M. hominis* antibody, which we find to be uncommon in young people and which reaches a maximum incidence in later life. This suggests that *M. hominis* does not behave as a respiratory pathogen or that, if it does, young people are not commonly infected.

Ante-natal patients, patients from general hospitals and patients of general practitioners are alike in antibody distribution. In comparison, antibodies to *M. hominis* are three times as common in female V.D. clinic patients and prison inmates. This is in accord with the results of other workers and may reflect the sexual promiscuity of these women. The overall incidence of growth-inhibiting antibody in male V.D. clinic patients and male hospital patients appears similar. This is partly explained by the difference in age distribution in the two groups and the high rate of positive results in hospital patients over 65.

SUMMARY

Sera from 3163 patients were examined for growth-inhibiting and complement-fixing antibody to *Mycoplasma hominis*. The results were analysed in respect of the age and sex of the patients. Antibodies were found to be uncommon in young people but increased in frequency with age; they were present in old men but were less common in women over sixty-five. Patients attending V.D. clinics and prison inmates had a much higher incidence of antibody than hospital and ante-natal patients. Complement-fixing antibody was approximately three times as common as growth-inhibiting antibody in the sample examined; it was also more common in women than men but the overall incidence of growth-inhibiting antibody was the same in each sex. It was concluded that possibly growth-inhibiting antibody is produced in more severe infections and that it disappears from the serum more quickly than complement-fixing antibody.

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The isolation of Coxsackie A viruses from human sera and mosquitoes in Fiji

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INTRODUCTION

In the middle and later years of the decade 1940–49 dengue was prevalent throughout the tropical islands of the Pacific. The inhabitants of Fiji were infected, a fact confirmed by a serological survey conducted in 1959–60 by Miles *et al.* (1964). Since 1950, illnesses diagnosed as dengue continued to appear, although at a much diminished rate. Illnesses diagnosed as influenza, however, were very prevalent both in epidemic years and in intervening periods. Evidence was lacking as to the proportion of these cases that were due to arboviruses, including dengue. Therefore, in 1963, attempts were made to isolate viruses from the sera of patients with febrile illnesses, and from mosquitoes caught both in dwelling houses and in the countryside.

Although no arboviruses were isolated, a number of strains of Coxsackie A 6 were isolated, both from sera of patients and from mosquitoes. The isolation of Coxsackie A 6 from sera has been reported before (Gear, Measroch & Prinsloo, 1956; Rodrigues *et al.* 1964), as has the isolation of Coxsackie viruses from mosquitoes (Taylor & Hurlbut, 1953), but such observations are relatively few.

METHODS

Virus isolations

The search for viruses was centred on Suva, which, with a population of 43,000, is the capital of Fiji, and is sited on the south-east corner of Viti Levu, the largest island of the group. The rainfall in this region is high, being about 130 in. per annum. The summer months, November to March, have a higher rainfall than the other months and are hot and humid. During these months mosquitoes are particularly plentiful.

During the last 3 weeks of January and the first week of February, liaison was established with the staff of a medical clinic, and samples of blood were taken at the clinic from patients suffering from ill-defined fever. Children in particular were the centre of attention.

Concurrently, mosquitoes were captured in the town and rural areas for the isolation of viruses. In the town, two Fijian mosquito inspectors visited houses

over a large area and collected, into sucking tubes, mosquitoes resting under the beds, in dark corners, wash-rooms, etc. In the rural areas the inspectors worked as a pair; one would remove his shirt and vest while the other with a sucking tube would suck off the mosquitoes alighting on him and preparing to bite.

After the mosquitoes or the blood from patients had been obtained they were taken to the laboratory in Suva. Sera were removed from the blood clots and stored at $-70°$ C. The mosquitoes were killed by freezing, and sorted into pools of approximately 100 according to species, date and place of capture. The pools were stored at $-70°$ C. until further processing, which consisted of trituration in buffered Hanks's solution, centrifugation at about 1000 r.p.m. for 10 min. and collection of the supernatant fluid.

Undiluted sera and the mosquito preparations were inoculated by the intracerebral and intraperitoneal routes into newborn mice. The mice were observed at least once daily for signs of illness and, if sick, they were killed and the brains removed for passage into further groups of mice.

The mosquito preparations were also inoculated on duck cell monolayers.

Serological survey

A limited number of serum samples collected from healthy children living on the three main islands of the Fiji group were available for testing for neutralizing antibodies. Intraperitoneal neutralization tests in 1-day-old mice were undertaken using a strain of Coxsackie A6 virus isolated from one of the patients in Fiji. If there was survival of more than two-thirds of the test-mice alive on the day following inoculation, the serum was considered positive for antibody content.

Table 1. *Age and sex of sixty patients examined for circulating virus*

	Age (years)						
	0–4	5–9	10+	20+	30+	40+	Unknown
Male	9	8	8	8	5	3	4
Female	6	3	3	1	0	2	
Total	15	11	11	9	5	5	4

RESULTS

Isolation of viruses from patients

Samples of sera were obtained from sixty patients whose age and sex distribution are shown in Table 1. Many of these patients complained of headache and body pains. Fever with a temperature of 100–102° F. was common. The cervical lymph nodes were frequently slightly enlarged and occasionally faucial injection was observed. No cutaneous eruptions suggestive of dengue were seen. Strains of virus were isolated from the sera of four patients (see Table 2). In every case re-isolation was successful. The four strains all had a similar behaviour in newborn mice, usually killing them in 3–4 days with widespread necrosis of the skeletal muscles, but with no appreciable damage to the brain or brown fat. They were

Table 2. *Sources and dates of virus isolations from human sera*

Specimen date	Race of patient	Age (years)	Sex	Days after onset	Remarks	Mouse inoculation: Date	Mouse inoculation: Days to death or sickness
8. i. 63	Part-European	8	F	0	Fever, 102° F, cough, tonsillar infection, total of 3 days illness	8. i. 63	1–3
14. i. 63	Indian	2½	F	0	Fever, 100° F, vomiting, ill for 3 days	14. i. 63	1–3
19. i. 63	Indian	8	M	1	Fever, 102° F, nausea cervical glands enlarged, complete recovery	21. i. 63	3
24. i. 63	Indian	6	M	1	Fever, 101·5° F, headaches and enlarged lymph glands	25. i. 63	3–10

Table 3. *Species and numbers of mosquitoes captured*

Species	Number
Aedes (*Aedimorphus*) *vexans*	1,653
A. (*Finlaya*) *fijiensis*	8
A. (*Ochlerotatus*) *vigilax*	4
A. (*Stegomyia*) *aegypti*	142
A. (*Stegomyia*) *polynesiensis*	36,007
A. (*Stegomyia*) *pseudoscutellaris*	43
Culex (*Culex*) *annulirostris*	4,187
C. (*Culex*) *quinquefasciatus*	1,505
C. (*Culex*) *sitiens*	3,273
Mansonia (*Coquilletidia*) *crassipes*	50
Total	46,872

Table 4. *Sources and dates of virus isolations from* Aedes polynesiensis *captured near Nukui village*

Pool no.	Date of capture	Date of mouse inoculation	Days to sickness or death of mice	Re-isolation
238	15. i. 63	3. v. 63	4	Not done
279	21. i. 63	23. viii. 63	2	Not done
369	22. i. 63	12. viii. 63	2	+
103	28. i. 63	7. ii. 63	4	+
233	28. i. 63	3. v. 63	5–7	+
237	28. i. 63	3. v. 63	6–8	+
305	28. i. 63	14. viii. 63	8	+
278	28. i. 63	19. viii. 63	5–7	−
267	28. i. 63	23. viii. 63	6	−

not inactivated by bile salts nor would they produce necrosis of duck embryo tissue cultures or HeLa cells. They were grouped as Coxsackie A viruses and subsequently shown by Dr L. Rosen to be type 6. This finding was confirmed in a neutralization test using specific monkey sera supplied by Dr J. Melnick.

Isolation of viruses from mosquitoes

The numbers and species of mosquitoes collected are shown in Table 3. Most of the *Aedes* (*Stegomyia*) *polynesiensis* were caught in rural areas, and the majority of the remaining species were caught in and around 100 dwelling houses in Suva. No viral isolations were made from mosquitoes caught in Suva, in spite of the fact that many contained undigested blood. Nine isolations in newborn mice, confirmed by re-isolation, were obtained from rural-caught *Ae.* (*S*) *polynesiensis*. The origin of these viruses is given in Table 4. All were derived from mosquitoes caught near the village of Nukui (lat. 18° 9′ S., long. 178° 34′ E.), situated among mangrove swamps at the southern tip of the Rewa delta. These strains have been identified as Coxsackie A 6.

Table 5. *Mouse neutralization tests on Fijian human sera using Coxsackie* A 6 (*V* 29 *strain*)

	Age group of donors								
	0–9 years			10–15 years			Total		
	No. tested	No. +	% +	No. tested	No. +	% +	No. tested	No. +	% +
Males	26	14	54	25	17	68	51	31	61
Females	19	12	63	13	12	92	32	24	75
Total	45	26	58	38	29	76	83	55	66

Serological survey

The results of the survey are shown in Table 5.

DISCUSSION

The clinical pictures of the infections were similar to those reported by Gear *et al.* (1956) and designated acute febrile lymphadenitis. Neither herpangina nor encephalitis was seen.

The isolation of the virus from the blood of four out of sixty patients suggests that an epidemic was occurring at that time, particularly when the rarity of viraemia in Coxsackie infections is recalled. Nevertheless the serological survey indicates that infections with Coxsackie A 6 virus are common in Fiji, presumably occurring in numerous small epidemics. Such a situation is to be expected with enteroviruses in tropical countries (Kalter, 1962; Rodrigues *et al.* 1964). The apparent difference in percentage of immunity between the sexes in our survey cannot be accounted for, but in any case it is hardly of statistical significance.

The frequency with which isolations were obtained from mosquitoes is striking,

and it is believed that they were not laboratory artifacts. Pools of mosquitoes and sera were stored in a low-temperature cabinet before processing, which occurred in a more or less random sequence. Some ensuing isolations were made when no previous isolations had been made for several months; and in the laboratory no grouping of isolations occurred. Yet on examining the source of the material a remarkable grouping is observed in the species of mosquitoes, and their times and place of collection. In fact all isolations were made from *Ae.* (*S*) *polynesiensis* caught near Nukui over a relatively short period.

The mosquitoes collected near Nukui could have become infected by biting the villagers or by biting the mosquito catchers who acted as bait. That the origin of the virus was the blood of the mosquito catcher is improbable for two reasons. First, the catchers took considerable care not to allow the mosquitoes, known vectors of filariasis, to probe. Secondly, the catchers remained symptomless throughout and even though symptom-free infections could have occurred it is unlikely that one or both could have carried circulating virus on four different days over a span of 14 days. Other vertebrates in the area were birds, rats and pigs and of these, pigs may have been a source of virus (Verlinde & Versteeg, 1958).

It appears that among different species of mosquitoes there is varying ability to harbour the virus. Nine isolations were made from *Ae.* (*S*) *polynesiensis*, whereas none were made from the other species captured. This could be due to the fact that only about a third as many of these others were captured. Nevertheless these were caught in houses in urban areas where the disease probably due to Coxsackie A 6 was known to be present and approximately 25 % of them contained blood.

At present it is impossible to assess the importance of the role of mosquitoes in transmitting this common infection. Further evidence is required on the degree and duration of viraemia in man and in particular on the ability of mosquitoes to transmit the virus rather than merely to harbour it. There are ample opportunities for the virus to pass from man to man other than by mosquito bites, so that in this respect mosquitoes may be of little importance.

SUMMARY

1. Coxsackie A 6 virus was isolated in January 1963 from the circulating blood of four children in Suva, Fiji, suffering from fever, lymphadenitis and pharyngitis.

2. Nine strains of the same agent were isolated from *Aedes* (*Stegomyia*) *polynesiensis* mosquitoes caught over a period of 2 weeks near or in a village situated among mangrove swamps at the mouth of the Rewa river, in January 1963.

3. No virus was isolated from mosquitoes caught in Suva.

4. A serological survey indicated that Coxsackie A 6 infections are common and widespread in Fiji.

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The incidence of glandular fever

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Published estimates of the incidence of illnesses described as glandular fever or infectious mononucleosis (regarded as synonymous in this paper) have varied enormously. The figures given have presumably been influenced by the methods used for diagnosis, by the type of community studied, and by the thoroughness of case finding. It is thus very difficult to know how frequently this disease presents in the general community. The present paper reports an attempt to determine the incidence of cases of proved glandular fever presenting to general practitioners in certain communities near Portsmouth, Hants.

Previous reports

Glandular fever has been notifiable in Bristol since 1960, and special efforts have been made there to find as many cases as possible. The apparent annual incidence of the disease has been increasing, and in 1963 and 1964 about 30 confirmed cases were found per 100,000 population (Medical Officer of Health, Bristol, 1960–64). Hobson, Lawson & Wigfield (1958) searched for cases in Oxford city with the co-operation of general practitioners, and diagnosed glandular fever on the basis of laboratory findings. They found an annual case incidence of 68 per 100,000 population (seronegative cases included). Oxford, however, is unrepresentative of the country as a whole, particularly in respect of glandular fever, because of the presence of large numbers of subjects (students and nurses) of an age especially liable to the disease. Those studying populations composed mainly of adolescent or young adult subjects find an even higher incidence; thus, Rugg-Gunn (1954), Evans (1960) and Yeager (1961) found annual rates equivalent to 171, 450 and 2000 respectively per 100,000 population.

Some reports deal with cases of glandular fever admitted to hospital. Ström (1960) in Stockholm and Belfrage (1962) in Malmö, Sweden, gave figures which indicate annual admission rates for this disease of about 23 and 30 per 100,000 population respectively (seronegative cases included) in 1955–57. Other reports deal with the incidence in the general community, the figures being derived from the number of positive serological tests for this disease recorded in laboratories. Thus, Newell (1957) reported an annual incidence of between 1·6 and 5·9 per 100,000 population in England and Wales. Virtanen (1962*a*, *b*), also using this approach, gave figures for Turku, Finland, indicating incidences of 4·5 and 6 per 100,000 population in 1960 and 1961 respectively. In neither Newell's nor Virtanen's work were special efforts made to find as many cases of glandular fever as possible, and Newell

acknowledged that his figures underestimated the incidence even of laboratory diagnosis of the disease.

Glandular fever became notifiable in Northern Ireland in 1949. The highest recorded annual incidence in that country up to 1962, however, was only 7·7 per 100,000 (Registrar General for Northern Ireland, 1949–1962). Glandular fever and infectious mononucleosis were among the diseases recorded by general practitioners from 106 practices in 1955–56 for the statistical study by the College of General Practitioners and the General Register Office. The 'patient consultation rate' for the two diseases together was 60 per 100,000 in the year (Logan & Cushion, 1958), but an incidence of 110 per 100,000 was recorded from certain selected practices in the south of England. Laboratory confirmation of the diagnosis was not required of doctors contributing to this study.

An altogether different incidence is suggested by the results of tests on the sera of healthy blood donors for the sheep-cell agglutinins typical of glandular fever. Barrett (1941), Hobson *et al.* (1958), and Virtanen (1962*b*) found such agglutinins in about 1 % of donor sera. Assuming that these agglutinins are rare except during and after glandular fever (Davidsohn, Stern & Kashiwagi, 1951), and that they persist for an average of three months (Kaufman, 1944; Davidsohn *et al.* 1951; Hoagland, 1952; Leibowitz, 1953), this finding implies that each year about 4000 per 100,000 blood donors have glandular fever with the transient appearance of sheep-cell agglutinins.

Not only is there dispute as to the overall incidence of glandular fever. The age incidence is also a point of disagreement. Most authors studying general populations and using laboratory methods of diagnosis find that only a quarter to a third of patients with glandular fever are aged less than 15 years (Press, Shlevin & Rosen, 1945; Stevens, Bayrd & Heck, 1951; Ström, 1960; Belfrage, 1962). Only 18 % of Newell's (1957) patients were in this age group. On the other hand, in the purely clinical study referred to above (Logan & Cushion, 1958) the incidence of the disease in those under 15 years was found to be 2½ times the incidence in those aged from 15 to 45. The discrepancy between the latter two findings is particularly striking since both the studies concerned covered England and Wales and were almost simultaneous. Such a discrepancy would occur if a clinical diagnosis of glandular fever in children is even more often mistaken than in adults.

The present investigation

A detailed investigation is now reported concerning the incidence of glandular fever in certain general communities near Portsmouth in 1962–63.

MATERIAL

The investigation was conducted in Petersfield urban district (together with certain hamlets in the surrounding rural district), in the town of Emsworth, and in the council housing estate of Leigh Park, north of Havant, Hants. The mid-1963 populations for these areas, as calculated from figures given by the local authorities, were 9450, 7800 and 27,550 respectively (figures to the nearest 50). Each of the areas is served by a weekly pathology clinic to which general practitioners refer

patients for blood tests. The work was carried out continuously for 12 months from October 1962.

Before the work started, all the general practitioners of the three districts were asked personally if they would let me know of any suspected cases of glandular fever; all agreed to do so. Most of these doctors were known personally to me, and would in any case have referred such cases. Patients were seen either at their homes, at the laboratory, or at the weekly clinics. Two patients originally referred with other diagnoses were found to have glandular fever and are included in the survey.

After the end of the year, exhaustive inquiries were made of all the general practitioners to try and trace any cases of glandular fever that might not have come to my attention. No such cases were traced, nor had any patient been admitted during the year, from the three areas, to the Portsmouth hospitals and been given a final diagnosis of glandular fever.

METHODS

A full history was taken and a physical examination made in all cases except five, in which time allowed only of brief details being taken (in these five, routine laboratory tests, only, were undertaken, but in none of them was the diagnosis of glandular fever confirmed). Venous blood was taken in all cases. Almost all patients were seen again between 10 days and 4 weeks later, and in most cases further specimens were taken. Some patients were seen yet again on later occasions.

Total leucocyte counts were carried out in both halves of a Neubauer counting chamber, and the process repeated if the counts differed by more than 10 % from their mean. Differential counts were made on 200 consecutive nucleated cells in blood films stained with the Knyvett–Gordon stain.

For the sheep-cell agglutinin test, doubling drop dilutions, up to 1/16, in physiological saline, were made of serum which had been inactivated at 55–56° C. for 30 min. Care was taken to minimize the carrying over in the pipette of excess fluid from tube to tube, and to see that any fluid on the side of a tube was well mixed with that at the bottom. To these dilutions, in one drop volumes in 0·5 × 5·0 cm. tubes, were added equal volumes of a 1–2 % suspension of packed, thrice washed, sheep cells in physiological saline, these cells being not less than 1 day and not more than 5 days old. The tubes were tapped to mix the contents, placed first in the 37° C. incubator for 1 hr. and then left at room temperature for 1 hr. They were read after gentle tapping, the highest dilution of serum giving macroscopic agglutination of the red cells being taken as the end-point. The reciprocal of this dilution, calculated after addition of the cell suspension, was the titre taken as the result.

If this test gave a titre higher than 4, or if a preliminary slide-screening test was positive, a full differential absorption test was carried out, using approximately 2 % guinea-pig kidney and ox-cell suspensions, and titrating with sheep cells as just described. All the sera were subsequently kept frozen, under sterile conditions, for further investigation; the sheep-cell agglutinin keeps well on storage (Bernstein, 1940; Davidsohn & Lee, 1964). At a later date, those sera which had shown

sheep-cell agglutinins persisting after guinea-pig kidney absorption were retested, using 20% guinea-pig kidney and ox-cell suspensions whose strengths had been checked by centrifuging. Three 0·7–0·8 × 17 cm. tubes were set up; into one were placed four drops of physiological saline, into another five drops of the ox-cell suspension, and into the third five drops of the guinea-pig kidney suspension. A drop of the inactivated serum was added to each tube, the contents gently shaken, and the tubes refrigerated overnight. Those containing antigen were then centrifuged, and doubling drop dilutions in saline made of the supernatants; corresponding dilutions were made of the serum–saline mixture. To each of the dilutions a drop of an accurate 2% suspension of sheep cells was added, and incubation and reading carried out as already described. No changes in the original diagnoses were made as a result of these further tests.

The methods for testing for sheep-cell agglutinins have been described in detail since results vary with the method used (Penman, 1966), and the value of many previously published results of this test has been lost because practical details were not given. Extensive serological, bacteriological and virological investigations were also carried out. The results of efforts to establish a diagnosis in patients shown not to have glandular fever will be the subject of another report.

RESULTS

In the three areas during the year, 53 patients were investigated in whom glandular fever had been suspected clinically, on the basis, *inter alia*, of apparent lymphadenopathy in an acute illness. Two other patients were found to have glandular fever, although this diagnosis had not originally been suspected.

In 17 of these 55 patients there was a mononuclear cell count of at least 4000/mm.^{3} (over 15,000/mm.^{3} in patients aged less than seven years) on at least one occasion, with a minimum of about 25% of these cells appearing atypical. These 17 patients were finally accepted as having had glandular fever. The sera from 13 of them showed sheep-cell agglutinins persisting after guinea-pig kidney absorption, with an overall absorption pattern typical of this disease (Davidsohn *et al.* 1951). The sera from two of the remaining four (one in Petersfield, one in Leigh Park) showed agglutinins persisting after guinea-pig kidney absorption in a special test, using an 0·2% sheep-cell suspension, with microscopic reading of the results. The two absolutely seronegative patients (one in Emsworth, one in Leigh Park) both showed mononuclear cell counts of more than 7000/mm.^{3}; the Emsworth patient showed a raised titre of antibody against *Toxoplasma gondii* (dye-test titre of 64), but, as there was no further rise in two later tests, the significance of this in relation to the current illness was doubtful. The 17 patients with glandular fever include a pupil at a Petersfield boarding school whose home was elsewhere, but exclude a patient whose home was in Petersfield but who suffered the disease away at his place of study. A nurse at St Mary's Hospital, Portsmouth, who returned home to Leigh Park after 12 days' illness, is included.

The geographical distribution of all 55 cases is shown in Table 1: 23 of the 38 (19 male, 19 female, all seronegative) patients shown not to have glandular fever

were aged less than 15 years, but only three of the 17 with glandular fever were in this age group. The four 'serogenative' glandular fever patients were aged 5, 33, 40 and 48 years respectively. The overall annual rate for glandular fever of 38 per 100,000 population means that, on average, one case might have been expected to have presented during the year in any one medium-sized general practice.

Table 1. *Occurrence of unconfirmed and confirmed cases of glandular fever in the three areas*

		Cases investigated		Cases confirmed	
	Mid-1963 population	Total	Annual rate per 100,000 population	Total	Annual rate per 100,000 population
Petersfield	9,450	13	138	6 (8*)	63 (56*)
Emsworth	7,800	13	167	5 (10*)	64 (85*)
Leigh Park	27,550	29	105	6	22
All three areas	44,800	55	123	17	38

* In Petersfield and Emsworth the occurrence of cases of glandular fever was recorded for a further 6 months; the figures in parentheses refer to the results at the end of the whole period of 18 months. The Emsworth figure of 10 includes three seronegative cases, one of which occurred in the first year of the investigation and is mentioned in the text.

The figures for the different areas, however, show that glandular fever apparently presented less frequently in the council housing estate than in either of the other two areas, where most houses are privately owned. This inconsistency may have arisen partly as a result of some cases in the housing estate not being referred for investigation. This possibility is discussed later, but it does seem that glandular fever really was less frequent in the housing estate, since the rate of referral of cases of suspected glandular fever from this area was in fact not much lower than the corresponding rate in the other two areas. The excess of patients with glandular fever in Emsworth and Petersfield did not seem to be accounted for by an undue number of mild cases presenting in these areas.

DISCUSSION

The figure of 38 per 100,000 population for the annual incidence of glandular fever in the general community seems rather low, particularly in relation to the figures, already quoted, for incidence calculated from serological tests on blood donor sera. This suggests either that the family doctors were not referring some of their cases for laboratory confirmation, that many patients ill with glandular fever do not consult their doctors, or that the disease frequently occurs in a subclinical form.

During both the present work and that of Hobson *et al.* (1958), some cases presenting to the family doctors may not have been referred for blood tests because, if the illness was atypical or of short duration, the possibility of glandular fever may not have been considered; even if it was, a venepuncture, particularly in children, may have been thought to be unjustified. In the present work the problem of atypical cases was covered as far as possible in that all the patients referred from

the three areas for blood tests for any reason were seen, and not just those suspected of having glandular fever. It is thought that all the patients in Emsworth and Petersfield suspected by their doctors of having glandular fever have been included in the series. In Leigh Park, however, a small fraction of the population (probably less than 5 %) was subsequently discovered not to have been included in the survey. This was because a few families, having moved from the city of Portsmouth to new houses on the estate, retained their old city doctors. Furthermore, in Leigh Park some of the doctors were less well known to me than those in the other two areas. Thus the real difference in incidence of glandular fever between Leigh Park and the other areas may be less than the figures in Table 1 suggest.

The number of patients whose glandular fever causes illness, but who do not consult their doctors, seems impossible to assess. Persons who for long periods do not consult their doctors appear to be healthy (Kessel, 1963), but this does not imply that the 'occasional patient' is perfectly well between consultations. Patients with mild glandular fever have been seen who, had they been so minded, might well not have consulted their doctors. Contratto (1944) and Bender (1958) found many such cases amongst students, and it seems probable that they are not infrequent in this country.

The possible existence of symptomless glandular fever has been investigated by means of haematological and serological examination of contacts of known cases of the disease. Evans & Robinton (1950) and Pejme (1964) have investigated this point thoroughly, but unfortunately obtained different results. The former authors found no good clinical, haematological or serological evidence of infection in contacts of five students who had had glandular fever between two and six weeks previously. Pejme (1964), however, found significantly more atypical cells in contacts of cases than in other healthy subjects, but he did not carry out serological tests on the contacts. Hoagland (1955) examined close contacts of patients with glandular fever and obtained negative differential agglutination results, though the blood of one contact showed 63 % lymphocytes, of which 1 % were atypical. It might be objected that, since glandular fever is not infectious in the usual sense of the term (Hoagland, 1955), contacts of patients might in any case not be expected to show signs of the disease. However, Hobson *et al.* (1958), on other grounds, thought that there was little evidence of subclinical glandular fever.

It seems then that the figure of 38 per 100,000 population for the annual incidence of glandular fever represents a minimum, Nevertheless, it is likely that, at least in the areas studied and during the time of the present investigation, the incidence of cases presenting to doctors did not greatly exceed this level. How much higher the true incidence might be seems impossible to assess.

The figure of 123 per 100,000 for the annual incidence of illnesses thought possibly to be glandular fever on clinical grounds is remarkably close to the rate of 110 per 100,000 for clinically diagnosed glandular fever found in certain practices in the south of England in 1955–56 (Logan & Cushion, 1958). The present figure of 123 is, in a sense, an over-estimate, for in a few cases in the present work diagnoses other than glandular fever subsequently became obvious on clinical grounds (e.g.

a case of measles). Such cases would not have been included in Logan & Cushion's figures. The present results show that a clinical diagnosis of glandular fever in a child is very likely to be incorrect.

SUMMARY

Previous estimates of the incidence of glandular fever vary widely. Reasons for this are suggested. The present investigation was designed to show the incidence of cases of glandular fever presenting to doctors in certain general communities near Portsmouth in 1962–63.

The overall incidence in these areas during the year was found to be 38 per 100,000 population; this amounts to an average of one case annually in a medium-sized general practice. The incidence appeared to be lowest in a council housing estate. Glandular fever was not common in children, although it was frequently diagnosed on clinical grounds.

The ways in which cases of glandular fever might be missed in a survey such as the present one are discussed. It is thought that many patients may become ill but not consult their doctors. Truly subclinical glandular fever, however, is thought to be infrequent.

This report is based on material included in a thesis accepted for the degree of M.D. at the University of Cambridge.

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The incidence of infective drug resistance in strains of *Escherichia coli* isolated from diseased human beings and domestic animals

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Following upon its discovery in Japan in 1959, infective resistance has been shown to be important in the spread of drug resistance among members of the Enterobacteriaceae; most of the available evidence has been reviewed by Watanabe (1963). The principal feature distinguishing infective resistance from other forms of drug resistance is that it can be transmitted from an organism of one species to an organism of the same or of a different species by contact. Recently, Smith & Halls (1966*a*) showed that most of the drug resistance in non-pathogenic *Escherichia coli* isolated from the faeces of healthy human beings, calves, pigs and fowls in Britain was infective and transmissible to the principal *Salmonella* serotypes and to some of the *E. coli* serotypes pathogenic for these species. Since non-pathogenic *E. coli* form the bulk of the enterobacteriaceal flora of the alimentary tract of man and domestic animals it is probable that these bacteria are the reservoir from which the numerically inferior pathogenic members of the group, e.g. *Salmonella*, *Shigella* and certain serotypes of *E. coli*, may acquire infective drug resistance.

As far as salmonellas are concerned, Datta (1962), Anderson & Datta (1965) Anderson & Lewis (1965) and Anderson (1965) have reported on the incidence of infective resistance in *Salmonella typhimurium* in man and domestic animals in Britain. Anderson & Lewis (1965), for example, found that 61% of 450 strains examined by them between December 1964 and February 1965 were resistant to one or more drugs; in a high proportion of these strains the resistance was of the infective type.

It is generally accepted that certain serotypes of *E. coli* are implicated in neonatal diarrhoea in human beings (Taylor, 1961), neonatal and post-weaning diarrhoea and bowel oedema in pigs and bacteraemia in fowls, calves and lambs (Sojka, 1965) There is not agreement as to the part *E. coli* plays in diarrhoea in calves and lambs. Antibacterial drugs are commonly used in the treatment of all these diseases. It seemed important, therefore, to determine the incidence of infective drug resistance in strains of *E. coli* isolated from babies and domestic animals suffering from these diseases; the results are reported in this paper.

MATERIALS AND METHODS

Escherichia coli *strains*

All the strains of *E. coli* were, as far as could be determined, epidemiologically unrelated. The human strains had been isolated from the faeces of babies suffering from neonatal diarrhoea in 1966. They had been sent by laboratories in Britain to Dr Joan Taylor of the Central Public Health Laboratory, Colindale, London, N.W.9, for serotyping; they belonged to serotypes considered enteropathogenic for human beings (Taylor, 1961). The strains from pigs had been isolated in 1960–62 or in 1965 from the small intestine or faeces of cases of neonatal or post-weaning diarrhoea or bowel oedema under conditions strongly suggesting that they were playing a pathogenic role. The fowl strains had been cultured from the internal organs of intensively reared broiler fowls that had died from 'coli-septicaemia', mainly in 1966. Of the 100 calf strains 14 had been isolated in 1962–65 from the internal organs of calves that had died from bacteraemia and the remainder from the small intestine or faeces of calves that had died from or were suffering from diarrhoea that could not be attributed to causes such as salmonella infection. Ten of the twenty-five lamb strains had been cultured from lambs that had died from bacteraemia and the remainder, under similar conditions as the calf strains, from cases of diarrhoea. The lambs, like the calves, were under 10 days old and were examined in 1962–65. The strains from the pigs, the fowls and those from the bacteraemic calves and lambs belonged to serotypes considered pathogenic for these species (Sojka, 1965).

Drug sensitivity tests

Approximately 0·15 ml. of a 1/50 dilution of a 24 hr. broth culture of the strain of *E. coli* to be tested was spread evenly over the surface of a dried MacConkey agar plate and disks containing different drugs were then applied at approximately equal distances apart. The amounts of drug used in the disks were 50 μg. of streptomycin, oxytetracylcine, chlortetracycline, chloramphenicol, neomycin and nalidixic acid, 25 μg. of ampicillin, cephalosporin C and polymixin, 15 μg. of furazolidone and 150 μg. of sulphadimidine. The plates were incubated at 37° C. for 24 hr. and read. Except in the case of sulphadimidine, no difficulty was experienced in making readings; the zones of inhibition surrounding the disks were either wide, very narrow or absent. All strains that appeared to be sulphadimidine-resistant were retested on nutrient agar in which the sulphonamide-inhibitors had been neutralized by the addition of lysed horse erythrocytes (Smith & Crabb, 1957). Similar results were obtained with oxytetracycline and chlortetracycline; in the results they are referred to collectively as tetracyclines. The results for cephalosporin C are not quoted because they were the same as those for ampicillin. Some tests were also performed with kanamycin and framycetin. They were not used as a routine because the results obtained with them were the same as those with neomycin.

Minimum inhibitory concentration of drugs for Escherichia coli

Nutrient agar plates containing twofold falling concentrations of the drug under test were inoculated lightly with 24 hr. broth cultures of strains of *E. coli* and incubated at 37° C. for 24 hr. The lowest concentration of drug that prevented growth was recorded as the minimum inhibitory concentration (MIC). Lysed horse erythrocytes were incorporated into the medium when sulphadimidine was studied and the cultures diluted so as to yield a growth consisting of discrete colonies on drug-free medium.

Transfer of drug resistance

Nutrient broth (Oxoid no. 2) in 10 ml. amounts was seeded with 0·02 ml. of a 24 hr. broth culture of the prospective donor strain of *E. coli*, i.e. one of those shown in sensitivity tests to be resistant to one or more drugs, and 0·1 ml. of a similar culture of the prospective recipient strain. In all the experiments in which the prospective donor strain was ampicillin-sensitive, an ampicillin-resistant chromosome mutant of a human enteropathogenic strain of *E. coli*, H 1, antigenic formula 026:K 60:H −, was used as recipient. This mutant was sensitive to all the other drugs studied. It had been obtained by heavily inoculating nutrient agar containing 30 μg./ml. of ampicillin with the fully sensitive H 1 and selecting one of the few colonies that grew thereon. The mixed culture of prospective donor and recipient strain was incubated at 37° C. for 24 hr. and then centrifuged. The deposit was inoculated on to a plate of MacConkey medium containing ampicillin and one of the drugs to which the prospective donor was resistant and the prospective recipient was sensitive (referred to as selection medium). The plate was incubated at 37° C. for 24 hr. Any colonies that grew on the medium were provisionally accepted as being those of the recipient strain to which drug resistance had been transferred from the donor strain; growth of the donor strain was suppressed by the ampicillin in the medium. One of the colonies was purified by plating on blood agar and a single colony picked into broth after it had been checked serologically to be of the recipient strain. The drug resistance of the resulting culture was then determined. Controls consisting of nutrient broth that had been inoculated with the recipient strain only were always kept. Whenever failure was encountered in attempts to transfer resistance to strain H 1, the relevant mixed culture experiment was repeated using an ampicillin-resistant mutant, prepared in the laboratory, from a drug-sensitive enteropathogenic pig strain of *E. coli*, P 11, antigenic formula 0141:K 85ab; 88ab, as recipient. This strain was also used in mixed culture experiments instead of H 1 when the prospective donor possessed an antigenic structure similar to that of H 1.

When the prospective donor was ampicillin-resistant, a nalidixic acid-resistant chromosome mutant of H 1, obtained in a similar manner as the ampicillin-resistant mutant, was employed as the prospective recipient, nalidixic acid being incorporated in the selection medium as well as one of the drugs to which the prospective donor was resistant. Finally the transmissible nature of the resistance of strains detected by the above methods was confirmed using a nalidixic acid-resistant

Table 1. *Incidence of drug-resistance in strains of* Escherichia coli *isolated from diseased human beings and domestic animals*

Strains	Source					
	Human beings	Pigs*		Calves	Lambs	Fowls
		1960–62	1965			
No. examined	70	70	70	100	25	35
Percentage resistant to one or more drugs	42·9	28·6	61·4	65·0	12·0	28·8
Percentage resistant to						
Tetracyclines	36·6	28·6	38·6	50·0	8·0	17·1
Streptomycin	35·3	11·4	40·0	52·0	8·0	2·9
Sulphonamides	33·0	8·6	22·9	57·0	12·0	17·1
Chloramphenicol	14·3	7·1	7·1	15·0	0	0
Ampicillin	18·6	0	0	14·0	4·0	0
Neomycin	4·3	0	7·1	5·0	0	0
Furazolidone	1·4	0	7·1	7·0	0	0
Polymixin	0	0	0	0	0	0
Nalidixic acid	0	0	0	0	0	0
Percentage with resistance pattern						
A S T C N Su	2·8	0	0	0	0	0
A S T C Su F	0	0	0	4·0	0	0
A S T C Su	5·7	0	0	1·0	0	0
S T C N Su	1·4	0	0	0	0	0
A S T Su	5·7	0	0	5·0	0	0
A S C Su	0	0	0	1·0	0	0
S T C Su	4·2	7·1	5·7	8·0	0	0
S T Su F	1·4	0	0	3·0	0	0
S T N Su	0	0	1·4	3·0	0	0
A S N	0	0	0	1·0	0	0
A S Su	1·4	0	0	0	0	0
A T Su	1·4	0	0	1·0	0	0
S T Su	4·2	1·4	5·7	16·0	8	2·8
S T N	0	0	2·8	1·0	0	0
S C Su	0	0	1·4	1·0	0	0
S Su F	0	0	1·4	0	0	0
S N F	0	0	1·4	0	0	0
T Su F	0	0	1·4	0	0	0
A Su	0	0	0	1·0	4·0	0
S T	5·7	2·8	2·8	2·0	0	0
S Su	2·8	0	5·7	5·0	0	0
S N	0	0	1·4	0	0	0
T Su	0	0	0	3·0	0	2·8
T F	0	0	1·4	0	0	0
A	1·4	0	0	0	0	0
S	0	0	10·0	1·0	0	0
T	4·2	17·1	17·1	3·0	0	11·4
Su	0	0	0	5·0	0	11·4
F	0	0	1·4	0	0	0

* The pig strains were classified according to the period of time in which they were isolated.

A, Ampicillin; S, streptomycin; T, tetracyclines; C, chloramphenicol; N, neomycin; Su, sulphonamide; F, furazolidone.

The results for cephalosporin C were the same as those for ampicillin. All the neomycin-resistant strains were resistant to kanamycin and framycetin.

chromosome mutant of *E. coli* K12F$^-$ as a recipient. In all studies employing sulphadimidine in the selection medium, the basal medium was nutrient agar in which the sulphonamide inhibitors had been neutralized in the manner described previously.

Normally, the drug levels used in the selection medium were 25 μg./ml. of streptomycin, oxytetracycline, chloramphenicol and neomycin, 30 μg./ml. of ampicillin and nalidixic acid, 10–15 μg./ml. of furazolidone and 1000 μg./ml. of sulphadimidine.

RESULTS

Incidence of drug-resistance amongst strains of Escherichia coli *isolated from diseased human beings and domestic animals*

The drug-resistance found amongst strains of *E. coli* isolated from human beings suffering from neonatal diarrhoea, calves and lambs suffering from neonatal diarrhoea or bacteraemia, pigs suffering from neonatal or post-weaning diarrhoea or bowel oedema and from fowls suffering from 'coli-septicaemia' is illustrated in Table 1.

The highest incidence of drug resistance was found amongst the strains from calves and the lowest incidence amongst those from lambs. The most complex resistance patterns were found amongst those from calves and human beings, some of the strains from these sources being resistant to as many as six drugs. In general, the incidence of resistance to any drug amongst the strains from any species appeared to be directly related to the extent that drug has been used in that species. The absence of neomycin and furazolidone resistance from the pig strains isolated in 1960–62 and their presence amongst those isolated in 1965 is probably a reflexion of the much greater usage of those drugs in pigs after 1962. It is noteworthy that the incidence of drug resistance amongst the 1965 pig strains was more than twice that in the 1960–62 pig strains. Of all the drugs studied, only two, polymixin and nalidixic acid, were active against all the strains of *E. coli* tested; both are drugs that have been used very little in human beings and not at all in domestic animals.

The incidence of infective resistance amongst the strains of Escherichia coli *isolated from diseased human beings and domestic animals*

The results of experiments designed to determine the incidence of infective resistance amongst a representative selection of the strains of *E. coli* from human beings and domestic animals referred to in Table 1 are summarized in Table 2. The resistance of most of the strains examined from all five species was of the infective type and was easily demonstrated to be so. The whole resistance pattern was usually transferable. In a minority of cases one or, very occasionally, two of the drug resistances involved in the complete resistance pattern of a strain was not transferred. Furazolidone resistance was exceptional; it was not transferred, despite repeated tests, from the eight furazolidone-resistant strains.

The ability with which resistance to different drugs could be transferred from mutliply and singly drug-resistant strains isolated in the present study is illustrated

in Table 3. Practically all the tetracycline, chloramphenicol and neomycin resistance was transmissible. This was so in the case of tetracycline resistance whether the donor was singly or multiply resistant; no strains resistant to chloramphenicol only or to neomycin only were found in this survey. Ampicillin resistance was

Table 2. *Incidence of infective resistance among strains of* Escherichia coli *from diseased human beings and domestic animals*

	No. of strains infectively resistant/no. of strains tested from:					
		Pigs				
Resistance pattern	Human beings	1960–62	1965	Calves	Lambs	Fowls
A S T C N Su	2/2	—	—	—	—	—
A S T C Su F	—	—	—	1/1 (A)	—	—
A S T C Su	4/4	—	—	1/1	—	—
S T C N Su	1/1	—	—	—	—	—
A S T Su	2/2	—	—	2/2	—	—
S T C Su	2/2	3/3	3/3	3/3	—	—
S T Su F	1/1	—	—	2/2	—	—
ST N Su	—	—	1/1 (S)	2/2 (S Su, S)	—	—
A S N	—	—	—	1/1 (A)	—	—
A S Su	1/1	—	—	—	—	—
S T Su	2/2	1/1	1/2	5/5 (T)	2/2	0/1
S T N	—	—	1/1	1/1	—	—
S C Su	—	—	1/1	—	—	—
S Su F	—	—	0/1	—	—	—
S N F	—	—	1/1	—	—	—
T Su F	—	1/1	—	—	—	—
A S	—	—	—	—	0/1	—
S T	3/3	1/1	—	—	—	—
S Su	0/1	1/1 (Su)	2/2	2/2 (Su)	—	—
T Su	—	—	—	—	—	1/1 (Su)
A	0/1	—	—	—	—	—
S	—	—	0/2	0/1	—	—
T	1/1	5/5	5/5	1/1	—	4/4
Su	—	—	—	1/2	—	1/3
F	—	—	0/1	—	—	—
Total	19/21	12/12	15/20	22/24	2/3	6/9

A, Ampicillin; S, streptomycin; T, tetracyclines; C, chloramphenicol; N, neomycin; Su, sulphonamide; F, furazolidone.

Furazolidone resistance was not transferred from any of the strains examined. The symbol of a drug, in parentheses, indicates that resistance to that drug was not transferred from *one* of the strains of the same resistance pattern that were examined, e.g. 5/5 (T) indicates that tetracycline resistance was not transferred from one of the five strains tested. Apart from these exceptions, the whole resistance pattern was transferred.

transferred from two-thirds of the strains tested. Infective resistance to streptomycin and sulphonamide was detected more commonly in multiply resistant strains than in singly resistant strains or in strains resistant to both these drugs only. As mentioned previously, none of the furazolidone resistance was transmitted.

To determine whether bacterial virus might be playing a mediating part in the transfer of drug resistance in the studies reported above, all the strains used as

donors were examined to see whether they were latently infected with bacterial virus active on the recipient strains. Only one donor strain was so infected. Its use did not result in the transference of drug resistance. To establish that the strains used as recipients were not unique or specially suited for the purpose, mixed culture experiments were performed using one tetracycline-resistant strain of *E. coli* as prospective donor and ampicillin-resistant or streptomycin-resistant chromosome

Table 3. *The ability to transfer single drug resistance from multiply and singly resistant strains of* Escherichia coli

	Kind of resistance possessed by prospective donor strains					
	Multiple		Streptomycin and sulphonamide resistance only		Single	
Drug resistance	No. tested	No. transferable*	No. tested	No. transferable	No. tested	No. transferable
Tetracycline	35	32	—	—	22	22
Chloramphenicol	12	12	—	—	—	—
Neomycin	7	7	—	—	—	—
Streptomycin	39	32	14	6	7	0
Sulphonamide	35	25	9	3	9	2
Furazolidone	7	0	—	—	1	0
Ampicillin	23	16	—	—	1	0

* No. of strains from which the particular resistance under study was shown to be transferable.

Table 4. *The minimum inhibitory concentration (MIC) of drugs for strains of* E. coli *classified as sensitive or resistant by disk tests*

	MIC (μg./ml.) for strains classified as					
			Resistant*			
	Sensitive		Natural		Transmitted	
Drug	Median	Range	Median	Range	Median	Range
Ampicillin	8	2–16 (10)	256	64–1024 (16)	512	64–1024 (11)
Cephalosporin C	4	4–8 (10)	256	128–256 (12)	—	—
Streptomycin	2	2–4 (20)	64	64–256 (20)	128	64–256 (20)
			> 2000	> 2000–> 2000 (9)	> 2000	> 2000 (4)
Oxytetracycline	4	4–8 (20)	512	64–512 (20)	512	64–1024 (17)
Chloramphenicol	4	4–8 (20)	512	256–1024 (20)	512	512–1024 (8)
Neomycin	2	1–2 (10)	256	128–1024 (9)	256	64–512 (7)
Sulphadimidine	20	10–40 (20)	> 5000	> 5000–> 5000 (20)	> 5000	> 5000–> 5000 (12)
Furazolidone	4	1–4 (20)	32	16–64 (7)	—	—
Polymixin	2	1–2 (20)	—	—	—	—
Nalidixic acid	4	2–4 (24)	—	—	—	—

* Most of the resistant strains classified as 'natural' were used as donors to produce from strain H 1 those classified as 'transmitted'.

The figures in parentheses refer to the number of strains tested.

Two levels of streptomycin resistance were encountered; the resistant strains are classified accordingly into two groups.

mutants of thirty-eight different strains of *E. coli* as prospective recipients. The selection medium employed contained oxytetracycline and ampicillin or streptomycin, depending on the mutant used as recipient. Each mixed culture experiment was performed once only. Tetracycline resistance was transferred to thirty-seven of the thirty-eight prospective recipient strains.

The minimum inhibitory concentration of drugs for Escherichia coli *strains classified as sensitive or resistant by disk tests*

The results of determining the minimum inhibitory concentration (MIC) of drugs for strains of *E. coli* that had been classified as sensitive or resistant by disk tests and of strains to which resistance had been transferred is summarized in Table 4. A great difference was noted between the MIC for strains that had been classified as resistant and for those that had been classified as sensitive; it was least in the case of furazolidone. The MIC of drugs for strains to which resistance had been transferred closely resembled those for the corresponding donor strains. Two levels of streptomycin resistance existed, both of which were transmissible.

DISCUSSION

The results show that most of the drug resistance possessed by the strains of *E. coli* isolated from human beings and domestic animals suffering from diseases of the alimentary tract or bacteraemia was of the infective type. This was also a feature of the non-pathogenic *E. coli* organisms that form the bulk of the enterobacteriaceal flora of the alimentary tract of these species (Smith & Halls, 1966*a*). The few failures to transmit resistance, other than those with furazolidone, were usually with ampicillin, streptomycin or sulphadimidine. Since chromosome mutants resistant to these three drugs could be obtained relatively easily from sensitive strains of *E. coli* by laboratory procedures, it is probable that the strains from which it was not possible to transmit resistance to these drugs were themselves mutants that had emerged as a result of selection pressure provided by drug-containing environments.

The high incidence of resistant strains to a large number of drugs and the complex resistance patterns of some of the strains was a disquieting feature of this survey, particularly as the diseases caused are acute and severe to the extent that they may terminate fatally if the drug with which they are first treated is not active against the infecting strain; the result of sensitivity tests cannot be awaited before commencing treatment. Only two drugs were found that were active on all the strains of *E. coli* tested. These were nalidixic acid and polymixin, drugs that are not commonly used in the treatment of *E. coli* infections. Since nalidixic acid-resistant mutants were easily obtained in the present work from cultures of *E. coli* by laboratory procedures, it is unlikely that nalidixic acid would have an extensive therapeutic life if brought into general use; the prospects of polymixin are better in this respect.

The results for the pig strains present a good illustration of the general situation that is developing as a consequence of the continued use of anti-bacterial drugs—

a doubling of the proportion of resistant strains between 1960–62 and 1965 with the presence in the 1965 collection of strains resistant to two drugs, neomycin and furazolidone, to which all the strains in the 1960–62 collection were sensitive. Since many of the multiply resistant strains from pigs and from the other species included tetracyclines in their resistance patterns, the incidence of these strains will be increased not only by the use in the treatment of clinical disease of any one of the drugs involved in the resistance patterns but also by such procedures as the continuous feeding with diets containing low levels of tetracyclines as growth promoters. The 'blanket' use of antibacterial drugs in flocks or herds in attempting to control non-specific 'stress' conditions or to stimulate egg production, policies which are powerfully advertised, is another potent stimulus to the emergence of drug-resistant bacterial populations. Nevertheless, it must be appreciated that a high incidence of drug resistance can occur when antibacterial drugs are used in a species solely for the treatment and prevention of clinical disease. This is well illustrated by the results for the human strains.

The broiler-fowl industry provides a good example of the influence the widespread use of antibiotic feeding has on the pathogenic *E. coli* in the animal population. From 1957 to 1960 the practice of feeding broiler fowls on diets containing tetracyclines increased greatly and progressively, and during that time Sojka & Carnaghan (1961) recorded the incidence of tetracycline-resistant strains amongst the pathogenic serotypes they isolated from broiler fowls suffering from 'coli-septicaemia' as 3·5 % in 1957, 20·5 % in 1958, 40·9 % in 1959 and 63·2 % in 1960. Since 1961 the three major animal food compounders, amongst others, have ceased adding tetracyclines to their broiler foods. In 1962 the incidence of tetracycline-resistant strains causing 'coli-septicaemia' had decreased to 30 % (K. M. Smith quoted by Smith, 1962) and in the present survey to 17·1 %.

The high incidence of drug resistance amongst the strains of *E. coli* examined from calves stems mainly from the frequent use of drugs in attempting to control diarrhoea in these animals. As mentioned previously, doubt arises as to the part *E. coli* plays in calf diarrhoea. Recent studies (Smith & Halls, 1966*b*) indicate that in only a small proportion of cases do these organisms play an aetiological role, most calf diarrhoea probably being non-infective in origin and the consequence of the harsh present-day methods of calf-rearing. The common practice of using antibacterial drugs in treating *all* cases of calf diarrhoea or in trying to prevent it, therefore, probably has little beneficial effect on calf diarrhoea as a whole but it gives rise to infectively-resistant non-pathogenic *E. coli* populations whose resistance may be transferred to strains that are truly enteropathogenic for the calf and to other pathogens such as *Salmonella*.

SUMMARY

A high incidence of drug resistance, mostly of the infective type, was found amongst strains of *Escherichia coli* isolated from human beings suffering from neonatal diarrhoea, calves and lambs suffering from neonatal diarrhoea or bacteraemia, pigs suffering from neonatal or post-weaning diarrhoea or bowel oedema and from fowls suffering from 'coli-septicaemia'. The strains from the

human beings, pigs and fowls, and those from calves and lambs suffering from bacteraemia, belonged to serotypes generally accepted as being pathogenic for these species.

Complex drug-resistance patterns were a common feature, particularly amongst the human and calf strains, some being resistant to six drugs. Only two drugs, polymyixin and nalidixic acid, were active on all the strains.

The incidence of drug resistance amongst pig strains was twice as great in those isolated in 1965 as in those isolated in 1960–62; neomycin and furazolidone resistance was found amongst the 1965 strains but not amongst the 1960–62 strains.

Infective resistance was easily demonstrated; tetracycline resistance was transferred from one tetracycline-resistant strain of *E. coli* to 37 of 38 tetracycline-sensitive strains at the first attempt.

Most ampicillin, streptomycin, tetracycline, chloramphenicol, neomycin and sulphonamide resistance was of the infective type; the furazolidone resistance in all of eight strains studied was not shown to be infective.

In the experiments to demonstrate the presence of infective resistance the level of drug resistance was usually found to be the same in donor and recipient strains.

I wish to thank Miss S. Harding and Mrs L. Stubbs for their capable technical help and Miss Sheila Halls, Mrs E. Johnson, Mr W. J. Sojka, Dr Joan Taylor and Mr J. R. Walton for advice and assistance in various ways.

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Further studies with quadruple vaccine

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Since our description of a trial with a new quadruple vaccine (Dane *et al.* 1962) against poliomyelitis, diphtheria, tetanus and pertussis there have been changes in the composition of the vaccine and in its recommended method of use in N. Ireland. During the past 2 years we have been concerned with finding the optimum schedule for infant immunization using quadruple vaccine, taking into account the immunological, medical and administrative factors involved, and also with methods of reducing the reactions caused by the pertussis component of the vaccine.

In this report we describe the results of a recent trial in which the quadruple vaccine used had a modified pertussis component. Immunization was started at 6 or 7 months of age in the majority of infants and was given in three doses, separated by intervals of 6–9 weeks and 6 months. The vaccines used were similar to those which have been in general use in N. Ireland since October 1964.

MATERIALS AND METHODS

In co-operation with Medical Officers of Health, parents' permission was sought to obtain blood samples from infants immunized at clinics with quadruple vaccine.

The vaccine. This was commercial 'Quadrilin' vaccine prepared by Glaxo Laboratories for use in N. Ireland, and differs in its pertussis component from 'Quadrilin' available elsewhere. The poliovirus-D-antigen content (Beale & Mason, 1962) was: type 1, 75 units; type 2, 2 units; type 3, 4 units. It also contained 28 Lf of diphtheria formol toxoid (F.T.), 5 Lf of tetanus toxoid and $12{\cdot}5 \times 10^9$ inactivated *Bordetella pertussis* (serotypes 1, 2, 4 and 1, 3) per 1 ml. dose.

The vaccination programme. Fifty-eight infants who had received no previous immunization took part in this trial. The volume of serum obtained from some of them did not allow all specimens to be tested against all antigens. The number of infants whose serum was tested against any particular antigen is shown in the relevant table of results. The age in months of the infants at the time they received their first dose of vaccine is shown in Table 1. The intervals in weeks between first and second doses and the intervals in months between second and third doses are shown in Tables 2 and 3.

Poliovirus-neutralizing antibody. A standard cytopathic test with approximately 100 TCD50 of virus was used (Dane, Dick, Briggs & Nelson, 1961). Antibody levels are expressed in terms of British standard units (Perkins & Evans, 1959).

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Diphtheria and tetanus antitoxin. Assay was by the methods described in the British Pharmacopoeia (1963 ed., pp. 1107, 1118).

Pertussis agglutinins. Agglutinin titres were estimated using a killed suspension of *B. pertussis*, composed of several strains of serotypes 1, 2, 4 and 1, 3. The antigen–serum mixtures were placed in a 37° C. water bath for 16 hr. before the test was read. Control antisera were included in each test. The antigen and control antisera were kindly supplied by Dr P. W. Muggleton of Glaxo Laboratories.

Table 1. *Ages of the fifty-eight infants immunized*

Months	5	6	7	8	9	10	11	12	13–36
No. of infants	2	21	15	5	6	2	2	2	3

Table 2. *Interval in weeks between first and second doses*

Weeks	4	5	6	7	8	9	10	11	12
No. of infants	1	2	12	11	9	19	2	—	2

Table 3. *Interval in months between second and third doses*

Months	5	6	7
No. of infants	3	45	10

Reaction follow-up studies. A Health Visitor visited the home of each infant on the day after inoculation. She recorded any reactions or possible reactions to the vaccine on a special form. If the infant had had a severe reaction such as persistent screaming or shock and collapse, she reported this to the laboratory and one of us (M.H.) visited the home, obtained a more detailed history and made a clinical examination of the infant.

RESULTS

Poliovirus antibody response

Blood samples for serological tests were taken approximately 1 month after the third and last dose of vaccine (see Table 4). The possibility cannot be excluded that at some time in their lives some of the infants may have had natural infections with polioviruses; such infections are however likely to have been uncommon, because during the study period there was no evidence that 'wild' polioviruses had been circulating in N. Ireland (J. H. Connolly, personal communication). In addition, only limited amounts of oral poliomyelitis vaccine had been used in the areas in which the children lived. Therefore we consider that the post-immunization neutralizing antibody titres (Tables 5, 6, 7) developed, in the majority of infants, as a result of immunization with quadruple vaccine. All infants developed antibody to all three types of poliovirus. The majority of titres recorded were very high, only two being $< 1/100$. These lower titres were both against the least important type II poliovirus.

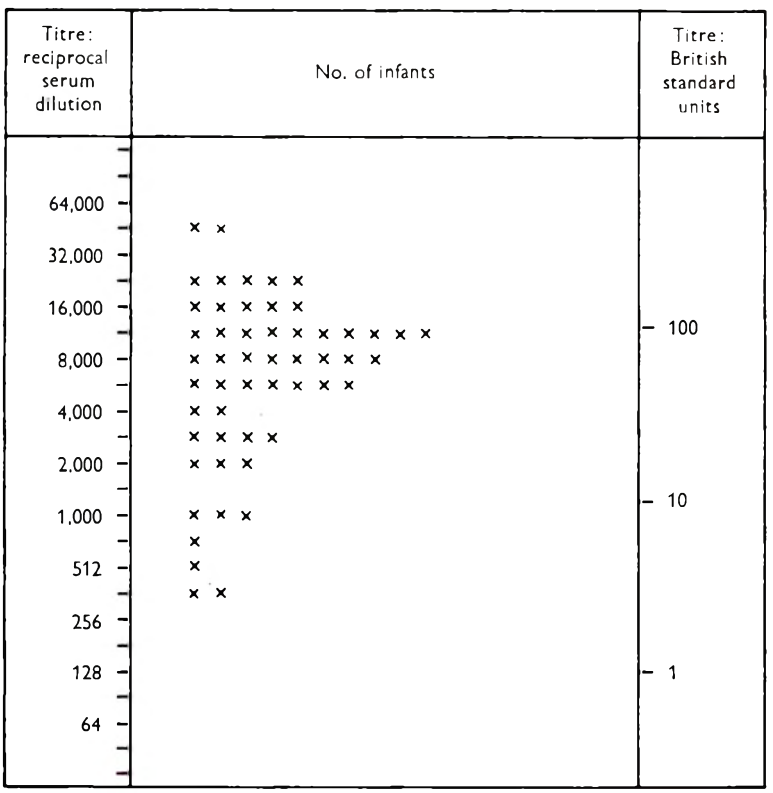
Diphtheria antitoxin response

The antitoxin titres after immunization in the serum of fifty infants are shown in Table 8. No clinical diphtheria has been recorded in N. Ireland since 1959; therefore these levels can be presumed to have resulted from immunization with quadruple vaccine.

Table 4. *Interval in weeks between third dose and obtaining serum sample*

Weeks	2	3	4	5	6	7	8	9
No. of infants	1	5	7	21	6	7	9	2

Table 5. *Type-I poliovirus-neutralizing antibody levels in fifty-three infants one month after the third dose of modified quadruple vaccine*



Tetanus antitoxin response

The antitoxin titres in the serum of fifty-eight infants after immunization are shown in Table 9. A few children had rather low titres, but these were well above the protective level of 0·01 units of antitoxin per millilitre of serum (Eckmann, 1963).

Pertussis agglutinins

In a Medical Research Council trial of pertussis vaccines (Report, 1956) it was shown that the agglutinin response of children was related to the field protective effect of the vaccines which had been prepared in the same way as the pertussis components of the various quadruple vaccines which we have studied. For this

Table 6. *Type-II poliovirus-neutralizing antibody levels in fifty-three infants one month after the third dose of modified quadruple vaccine*

Titre: reciprocal serum dilution	No. of infants	Titre: British standard units
32,000		
	× ×	
16,000	× × ×	
	× ×	
8,000	× × × × × ×	
	× × × ×	100
4,000	× × × × × × × × ×	
	× × × × ×	
2,000	× × ×	
	× ×	
1,000	× × × × × × ×	
	× × ×	
512	× × ×	10
256		
	×	
128	×	
64		1
32		
16	×	
8	×	

reason the likely effectiveness of the vaccine was measured by means of the agglutinin response and comparisons were also made between the agglutinin responses recorded in this trial and those from previous trials. The agglutinin responses of the infants in the present trial are shown together with results from two previous trials in Table 10. It will be seen that, despite the fact that the total number of organisms given in the present schedule (trial C) was less than half that given in trial A, in which the three doses of quadruple vaccine were spaced at monthly intervals, the geometric mean titre (GMT) was considerably higher in trial C. The GMT for the infants in trial B was somewhat higher than for those in trial C, but four doses had been given and a total of three times as many pertussis organisms.

Reactions

After the introduction of quadruple vaccine in N. Ireland in 1963 reports were received that reactions, similar to those which are known to follow the administration of vaccines containing inactivated *B. pertussis* (Hopper, 1961), were occurring with sufficient frequency to influence the acceptance of the vaccine. In order to obtain a clear picture of the type, severity and frequency of the reactions a follow-

Table 7. *Type-III poliovirus-neutralizing antibody levels in fifty-three infants one month after the third dose of modified quadruple vaccine*

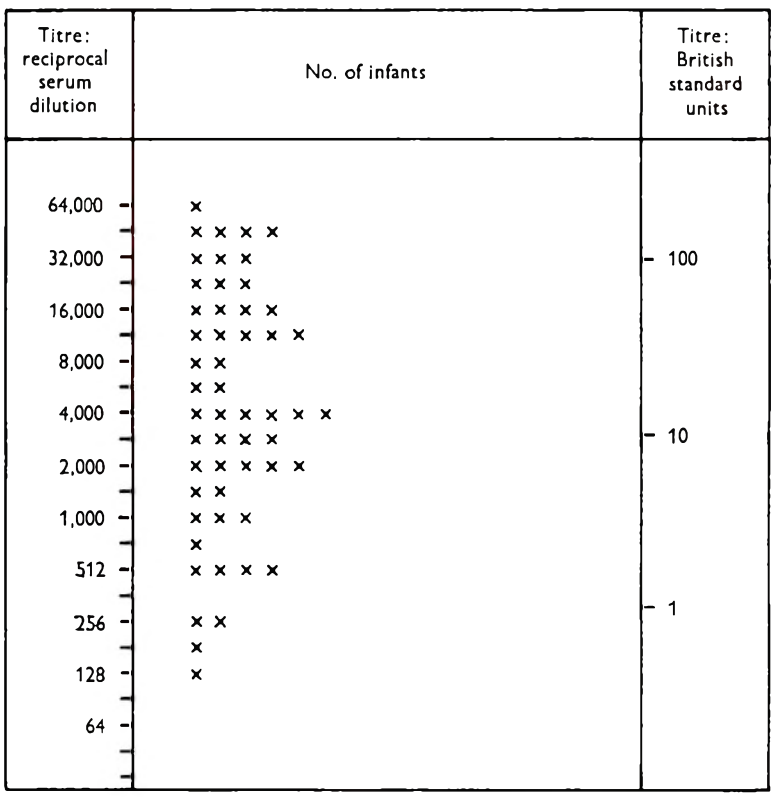


Table 8. *Diphtheria antitoxin levels in fifty infants one month after the third dose of modified quadruple vaccine*

Diphtheria antitoxin units per ml. serum	No. of infants
< 0·1	0
0·1–1·0	6
1·0–10·0	15
> 10·0	29

up study was arranged in co-operation with Medical Officers of Health and Health Visitors. The manufacturers were asked to produce a trial batch of quadruple vaccine in which the pertussis component was prepared in a different manner and

the total number of organisms reduced. The reactions to this modified vaccine and to the standard vaccine were then compared in a double-blind trial. The modified vaccine, which had passed the usual potency tests (British Pharmacopoeia, 1963 ed., p. 1122) was found to produce significantly fewer and milder reactions than the standard vaccine. Production batches of this modified vaccine have been in general use in N. Ireland since October 1964, and the infants immunized in the present study received only modified vaccine. A recent follow-up study of 400 infants immunized at routine clinics has shown that reactions are less severe and less

Table 9. *Tetanus antitoxin levels in fifty-eight infants one month after the third dose of modified quadruple vaccine*

Tetanus international antitoxin units per ml. serum	No. of infants
0·1	0
0·1–0·2	2
0·2–0·4	1
0·4–0·8	3
0·8–1·6	7
1·6–3·2	6
3·2–6·4	26
6·4–12·8	6
12·8	7

Table10. *Pertussis agglutinin titres (reciprocal of serum dilution) for infants in three quadruple vaccine trials*

	Trial A	Trial B	Trial C*
No. of doses given . . .	3	4	3
Approximate age for each dose (months) . . .	6, 7, 8	3, 4, 5, 12	6, 7½, 14
No. of organisms per dose ...	29×10^9	29×10^9	$12{\cdot}5 \times 10^9$
No. of infants in trial ...	31	60	58
		No. of infants	
Pertussis agglutinin titres			
<4	3	—	—
4	1	—	—
8	1	—	—
16	1	—	—
32	2	—	—
64	3	2	1
128	2	3	14
256	11	30	22
512	5	15	15
1024	2	10	1
2048	—	—	—
4096	—	—	5
Geometric mean titre ...	96	354	259†

* The present trial.

† The five infants with titres of 1/4096 have been omitted from the GMT calculation because they possibly had natural infections.

frequent than in the past. This was shown clearly when the reactions recorded for 250 infants who had received former production batches of quadruple vaccine were compared with those for 250 infants of the same age and sex who had received the modified vaccine (Table 11). Satisfactory reports have also reached us from the M.Os.H. using the modified vaccine indicating that reactions have been greatly reduced and that vaccine acceptance has been good.

Table 11. *Comparison of incidence of reactions following the original 'Quadrilin' vaccine and the modified 'Quadrilin' vaccine now used in Northern Ireland*

(Total numbers are given. Percentage figures are given in parentheses.)

	Original quadrilin	Modified quadrilin
No. observed	250	250
Mild reactions		
Fretful	176 (70·4)	78 (31·2)
Flushed and feverish	75 (30·0)	22 (8·8)
Drowsy	28 (11·2)	13 (5·2)
Skin rash	8 (3·2)	0
Painful arm	140 (56·0)	39 (15·6)
Swollen arm	83 (33·2)	16 (6·4)
Off food	38 (15·2)	10 (4·0)
Severe reactions		
Persistent screaming	12 (4·8)	1 (0·4)
Shocked or collapsed	3 (1·2)	0
No reported reactions	41 (16·4)	149 (59·6)

Table 12. *The age of infants in all quadruple vaccine follow-up studies who had severe reactions*

Age	No. of severe reactions*	Total observed
Under 6 months	23	498
Over 6 months	1	471

* Persistent screaming, and shock and collapse.

Though no direct comparison was made between the reactions following triple (diphtheria, tetanus, pertussis) vaccine and those following the quadruple vaccines, similar follow-up studies have been done. Two triple vaccines made by different manufacturers had quite different reaction rates. One was comparable to the modified quadruple vaccine, but the other gave rise to reactions of much the same order of frequency and severity as the original quadruple vaccine.

The severe type of reaction such as persistent screaming and shock and collapse (Hopper, 1961) was found to be more common amongst younger infants, and the large majority of the severe reactions were in infants under the age of 6 months (Table 12).

A detailed account of these follow-up studies of reactions to quadruple and triple vaccines will be published shortly.

DISCUSSION

The serological response to the poliovirus antigen in the modified quadruple vaccine was highly satisfactory, particularly against the important type I poliovirus component. The type I antibody titres were rather higher than those for types II and III and as a consequence the manufacturers have recently approximately doubled the 'D' antigen content for types II and III. It seems probable that, if the first dose of quadruple vaccine was given at 4 or 5 months of age, instead of at about 6 months of age as in the present trial, a satisfactory serological response would be obtained with three *suitably spaced* doses of the vaccine despite the relative immunological immaturity and higher levels of passive maternal antibody present at this earlier age. Our own reservation about starting immunization before the age of 6 months with vaccines containing a pertussis component are based in part on the greater toxicity of that component of the vaccine for younger infants.

The antitoxin responses to diphtheria and tetanus toxoids were satisfactory. Several infants had rather low levels of circulating tetanus antitoxin and the general level of response could be raised by increasing the amount of toxoid (5 Lf) present in the vaccine. However, if the present amount is adequate there seems little point in increasing it, as even tetanus toxoid may occasionally cause reactions (Brindle & Twyman, 1962; Eisen, Cohen & Rose, 1963).

Though the agglutinin response to *B. pertussis* is a less satisfactory way of measuring protection indirectly than the methods used for assessing the effectiveness of the other components it is of some value (Report, 1956) and comparisons made between results following different immunization schedules are probably valid. The fact that better results were obtained with modified quadruple vaccine given in three doses spaced 6–9 weeks and 6 months apart than with the original quadruple vaccine given in three doses at monthly intervals, though the original vaccine contained more than twice the number of pertussis organisms, is almost certainly a reflexion of superior dosage spacing. When sufficient time is allowed before the third dose is given there is a good secondary antibody response. In fact immunization with pertussis antigen seems no different from immunization with many other antigens such as poliovirus or tetanus toxoid where this type of dosage spacing has been accepted for some time. The majority of children, in our experience, receive triple vaccine (diphtheria, tetanus, pertussis) in three doses at monthly intervals, though in fact in both schedules P and Q of the Ministry of Health (Ministry of Health, 1961) it was recommended that there should be an interval of about 6–12 months before the last dose was given. We believe therefore that quadruple vaccine given in the present schedule could do much to raise the level of artificially induced pertussis immunity in young children at the age of maximum incidence of the disease.

For those who prefer giving three doses of triple vaccine and at the same time oral poliovaccine as an alternative to quadruple vaccine we would recommend that serious consideration be given to a similar type of dosage spacing to that used in the present trial, even if immunization is started at 4 or 5 months of age. Admini-

stratively it is somewhat easier to give three doses at monthly intervals, but in this instance administrative factors should probably not outweigh immunological factors. We have found that a three-dose schedule is much more acceptable than a four-dose schedule and is more likely to be completed.

We were surprised to find the frequency of reactions such as persistent screaming and shock and collapse that occurred not only after the original quadruple vaccine but also after some commercial triple vaccines. Hopper (1961) noted these reactions but we think that it was not generally appreciated how common they were. It has been obvious from our recent follow-up studies of quadruple vaccine that infants under 6 months of age are much more frequently subject to severe reactions than those over 6 months (Table 12). The fact that the majority of infants in our original trial of quadruple vaccine and also those in the Medical Research Council whooping-cough vaccine trials (Medical Research Council, 1951; Report, 1956; Report, 1959) were over 6 months of age may have been partly responsible for this ignorance.

The methods adopted to overcome the problem of too frequent and severe reactions were firstly to alter the technique of preparing pertussis antigen to one that was thought to produce a less reactogenic but equally protective vaccine, and secondly to reduce the number of pertussis organisms in each dose.

The reduction in the number of pertussis organisms did not in fact appear to reduce the infants' agglutinin response appreciably (Table 10), though unfortunately no direct comparison was possible between the original and the modified quadruple vaccines using the same dosage schedule. In our opinion any slight loss of antigenic potency which has followed the reduction in the number of pertussis organisms present in quadruple vaccine has been fully offset by the reduction in the severity and frequency of reactions and by the increased acceptability of the vaccine.

SUMMARY

Reactions to the pertussis component of the original commercial batches of quadruple vaccine against poliomyelitis, diphtheria, tetanus and pertussis (Quadrilin, Glaxo Laboratories) gave some cause for concern. Severe reactions were found to be more common in infants under 6 months of age than in older infants.

A modified quadruple vaccine, which has been used in N. Ireland since October 1964, was found to give rise to significantly fewer and milder reactions. This vaccine when given in three doses separated by intervals of 6–9 weeks and 6 months to fifty-eight infants most of whom were 6–7 months of age at the start of immunization was found to give a satisfactory immunological response.

We wish to thank the Medical Officers of Health and Health Visitors of Belfast, County Down and County Antrim for their co-operation; the children and their parents; and Mr J. J. McAlister, Miss Ffiona Wells, Mr K. Johnston, Mr R. J. Cummings and Mr A. W. P. Stevens for technical assistance.

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Further studies with a diphtheria–tetanus–poliomyelitis vaccine

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In 1965 we reported a trial of a combined diphtheria, tetanus and poliomyelitis (dip./tet./pol.) vaccine which had been designed for reinforcing the immunity of children at the time they entered school (Dane *et al.* 1965). One dose was found adequate for this purpose in children who had been previously immunized in infancy. The Ministry of Health (1965) recommended that either a single dose of dip./tet./pol. vaccine or a dose of oral poliovaccine and a dose of diphtheria/tetanus vaccine be used for reinforcing immunity at school entry.

From an administrative point of view there would be obvious advantages if *all* children were given one dose of dip./tet./pol. vaccine at school entry, irrespective of their past immunization history. Those with no history of previous immunization could then be given two further doses of the same vaccine to complete their primary course. The trial described here was designed to investigate the use of dip./tet./pol. vaccine for primary immunization in this way. We did not initially recommend this procedure because of doubts about the efficiency of diphtheria formol toxoid when used in a vaccine containing no adjuvant, such as *Bordetella pertussis* or a mineral carrier, though we considered the other two components would provide adequate immunity.

MATERIALS AND METHODS

In co-operation with parents and the Belfast County Borough Health Department we immunized thirty-nine school-children aged 5–6 years selected because they were thought not to have had any previous immunization against diphtheria or tetanus. Blood samples were taken at the time of the first injection and again one month after the third.

The vaccine. Commercial dip./tet./pol. vaccine prepared by Glaxo Laboratories was used. Each 1 ml. dose contained 56 Lf of diphtheria formol toxoid and 10 Lf of tetanus toxoid. The poliovirus D-antigen content (Beale & Mason, 1962) was: type 1, 75 units; type 2, 3 units; type 3, 6 units. The vaccine was given in three intramuscular doses with intervals of 6 weeks between the first and second and 6 months between the second and third doses.

Diphtheria and tetanus antitoxin. Assay was by the methods described in the British Pharmacopoeia (1963 ed., pp. 1107, 1118).

Poliovirus-neutralizing antibody. A standard cytopathic test employing approxi-

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mately 100 TCD50 of virus was used. The titres of British Standard antisera (Perkins & Evans, 1959) were: type 1, 1/1500; type 2, 1/650; type 3, 1/3000.

RESULTS

Diphtheria and tetanus

Five of the thirty-nine children were found to have low levels of either diphtheria or tetanus antitoxin in their pre-immunization blood samples. The antitoxin levels in the blood of the remaining thirty-four children 1 month after the third dose of vaccine are shown in Tables 1 and 2.

Table 1. *Diphtheria antitoxin levels in thirty-four children after three spaced doses of dip./tet./pol. vaccine*

(The children had no previous history of immunization, and all had titres of < 0·001 units per ml. serum before receiving the vaccine.)

	Antitoxin units per ml. serum		
	0·05–0·10	0·10–1·0	1·0–10·0
No. of children	3	13	18

Table 2. *Tetanus antitoxin levels in thirty-four children after three spaced doses of dip./tet./pol. vaccine*

(The children had no previous history of immunization, and all had titres of < 0·02 units per ml. serum before receiving the vaccine.)

	International antitoxin units per ml. serum					
	0·4–0·8	0·8–1·6	1·6–3·2	3·2–6·4	6·4–12·8	> 12·8
No. of children	1	1	9	10	10	3

Table 3. *Poliovirus-neutralizing antibody levels in thirty-nine children who received three spaced doses of dip./tet./pol. vaccine*

(A = Pre-immunization serum; B = post-immunization serum.)

	No. of children					
	Type I		Type II		Type III	
Reciprocal of serum antibody titre	A	B	A	B	A	B
< 10	6	0	1	0	4	0
10–100	4	0	3	0	3	0
> 100–1000	13	1	14	2	13	2
> 1000	16	38	21	37	19	37

Poliomyelitis

Though the majority of children in this trial had no previous immunization against diphtheria or tetanus, many had been immunized against poliomyelitis; therefore little information could be obtained about the effectiveness of dip./tet./

pol. vaccine for *primary* immunization against this disease. The neutralizing antibody levels of the children before and after immunization are shown in Table 3.

DISCUSSION

The efficiency of diphtheria formol toxoid as an immunizing agent when given with an adjuvant is well established (Report, 1959). However a report to the Medical Research Council (Report, 1962) indicated that highly purified, plain diphtheria formol toxoid is a relatively poor antigen, unsuitable for primary immunization and its use either alone or in combination with tetanus toxoid has not been recommended by the Ministry of Health since 1963 (Ministry of Health, 1963). In the present trial the post-immunization levels of diphtheria antitoxin were found to be adequate even though the dip./tet./pol. vaccine contained no adjuvant. Two factors may account for this apparent difference in efficiency between plain diphtheria formol toxoid and this antigen as a component of dip./tet./pol. vaccine. First, the diphtheria toxoid in combination with two other components as in dip./tet./pol. vaccine is less pure, and, secondly, the dip./tet./pol. vaccine was given in three doses separated by intervals of 6 weeks and 6 months whereas the diphtheria toxoid was given in a more closely spaced schedule of two or three doses 4–6 weeks apart.

The response to the tetanus component of the vaccine was satisfactory. This had been expected because, even without adjuvant, tetanus toxoid is suitable for primary immunization when given in three suitably spaced doses (Boyd, 1959).

We had no reason to believe that present-day potent inactivated poliovirus antigens would be unsuitable for primary immunization and therefore no attempt was made to select children for the trial who were devoid of poliomyelitis antibody. The majority of children had moderate or high levels of neutralizing antibody to at least two of the poliovirus types before immunization with dip./tet./pol. vaccine. As expected, after the third dose of vaccine all children had high levels of antibody to all three poliovirus types.

Though the serological responses of the children in this trial suggest that dip./tet./pol. vaccine is suitable for primary immunization of children at 5 or 6 years of age, this cannot be taken as evidence that it is also suitable for immunizing very young infants. When, for one reason or another, it is thought undesirable to give an infant a pertussis-containing vaccine then dip./tet./pol. vaccine would probably be effective provided that it is given after the age of 6 months, when the inhibitory influences of maternal antibody and immunological immaturity have waned (Evans & Smith, 1963).

It might be argued that dip./tet./pol. vaccine could be improved by the addition of a mineral-carrier adjuvant. Such an addition would result in a better response to the diphtheria toxoid but it might also lead to more local reactions. In its present form the vaccine causes negligible reactions (Dane *et al.* 1965) and we consider that it would be unwise to alter it in a way which might decrease its acceptability.

The administrative convenience of being able to use a single vaccine for protect-

ing young school-children against diphtheria, tetanus and poliomyelitis, whether they have had previous immunization or not, is considerable. There was a 6 months interval between the second and third doses in the present trial but if this was extended to $10\frac{1}{2}$ months it would be possible to give third doses, at the same time as first doses were being given to next year's intake of children into a school. In this way a school Medical Officer making two visits to a school a year and using a single vaccine could ensure that adequate continuing immunity was provided against diphtheria, tetanus and poliomyelitis.

SUMMARY

The efficiency of a diphtheria, tetanus, poliomyelitis vaccine in inducing a serological response after a three-dose primary course of immunization was tested in thirty-nine children aged 5 and 6 years and found to be satisfactory. This vaccine had previously been shown to be suitable for use as a single dose reinforcing vaccine for children of this age who had been immunized in infancy. It is suggested that all children might receive one dose of the vaccine at the time they enter school, and then those who have not been immunized before should receive a further two suitably spaced doses to complete their course of primary immunization.

We wish to thank Dr W. J. McLeod, Deputy Medical Officer of Health of Belfast; the children and their parents; and Mr J. J. McAlister, F.I.M.L.T., Miss Ffiona Wells, Mr T. J. Cummings and Mr A. W. P. Stephens for their technical assistance.

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A quantitative and qualitative appraisal of microbial pollution of water by swimmers: a preliminary report

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When a person goes swimming, micro-organisms are washed off the skin and out of some body cavities and into the water, polluting it to an unmeasured degree. The normal microbial flora of the human body has been studied by many investigators and has been reviewed comprehensively by Rosebury (1962). The so-called indigenous biota varies from person to person to environment. The organisms, commonly known as pathogens for man, may or may not be present in a healthy person, an individual having a subclinical infection, or a convalescent. All such possibilities have been taken into consideration by public health officials as they have promulgated standards for the bacterial quality of swimming pool waters. Although there has been only limited epidemiologic evidence which has directly related disease with swimming, the possibility is always present.

The lack of these data concerning the disease potential of swimming in polluted water has caused public health and regulatory agencies to use drinking water standards, with some modification, as indices of acceptability. Thus, it has been customary to use chemical disinfectants, usually chlorine or its compounds, in the treatment of swimming pool waters and to apply the same indices of pollution, namely coliform and/or faecal streptococcus groups of bacteria. The use of these indicators has been challenged over the years by many persons, such as Seligmann (1951), Mallman (1928, 1962), and more recently by Favero, Drake & Randall (1964), all of whom believe that cocci represent more correctly the actual bacterial quality of the pool water since large numbers of body cocci are washed into the water. Some of these organisms, such as staphylococci, are moderately resistant to chemical disinfection. McLean (1963) has pointed out that these microbes may be excreted into the water from the skin, and from mouth and nose by sneezing, coughing, and blowing water out of the mouth. Although these practices are discouraged, it is known that they commonly occur. The widespread, almost universal, requirement that a shower be taken before the swimmer dons his suit and enters the pool is intended to reduce the amount of microbial pollution, but the validity of this practice has not been studied thoroughly.

The present study was undertaken to determine, if possible, characteristics of

microbiological pollution that a female swimmer might add to filtered water without residual disinfection. This was a preliminary attempt to determine the major types of bacteria being shed under varying conditions of individual personal hygiene, including those during the menstrual period.

MATERIALS AND METHODS

Design of study

A special swimming tank was designed to permit an adult swimmer adequate width and depth to simulate a back stroke, a crawl stroke, and a breast stroke while 'afloat' in 150 gallons of water. The tank was constructed of fibre glass which had been polished to the smoothness of glass and which contained neither seams nor other areas where bacteria might lodge. The design of the tank is shown in Fig. 1.

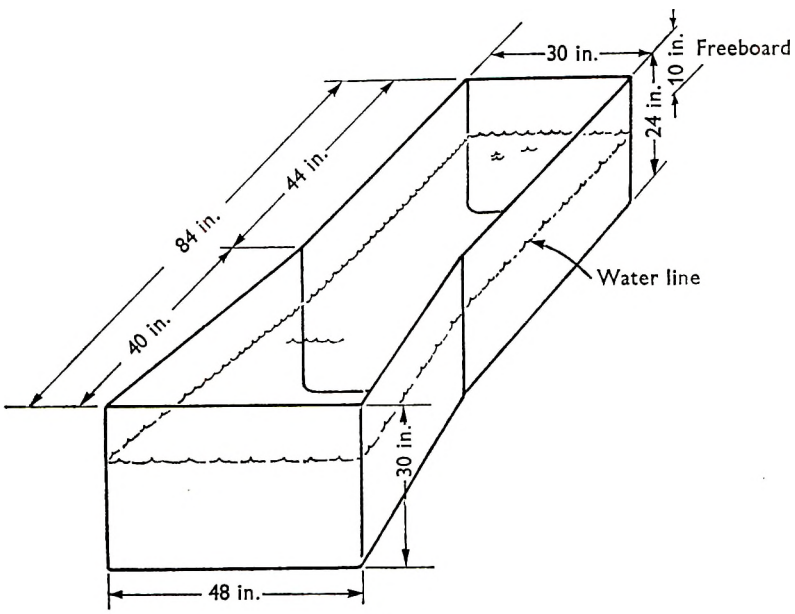

Fig. 1

Water was obtained from the regular city supply. No chlorine residual was ever demonstrated with an amperometric titrator. The water was drawn from a mixing faucet at a temperature of 80° F. through a cartridge-type filter and then directly through nalgene tubing into the experimental tank immediately before each test run.

Five adult females actively engaged in the teaching of physical education, ranging in age from 25 to 45 years, were selected as test subjects. Each individual was asked to maintain her normal daily routine of personal hygiene. Personal data on these subjects are given in Table 1. Four of these individuals took daily tub baths, while one showered daily. Three routinely used brand D soap, one used brand CB, and one used brand I. Three subjects used no skin lotions or special cosmetic preparations; one used brand N, and one used brand P, in daily face washing.

The subjects participated in this experiment during their menstrual period with and without the use of tampons in an attempt to determine whether or not there was any noticeable difference in the bacterial pollution of the water.

When a shower was indicated as part of the experiment, each subject took a hot shower using brand I soap, followed by thorough rinsing. All subjects wore sterile, one-piece cotton bathing-suits and a standard rubber swim-cap; and they wore sterile plastic boots from the locker/shower-room area to the test area.

Table 1. *Personal hygiene data on five female subjects*

Subject	Usual mode of daily bathing	Soap used daily	Skin lotion used	Type of skin	Usual menstrual flow
A	Tub bath	D*	None	Oily	Moderate
B	Tub bath	CB	None	Normal	Moderate
C	Tub bath	D*	N	Normal	Heavy
D	Tub bath	D*	None	Dry	Moderate
E	Shower	I	P*	Normal	Moderate

* Contains hexachlorophene.

The first water sample for bacteriological examination was taken just before the subject entered the tank. The subject, after removing her plastic boots, entered the tank and, supported by a nylon strap, began the series of simulated swimming, 5 min. of the back stroke, 5 min. of the crawl, and 5 min. of the breast stroke.

A sample of water was taken immediately upon conclusion of the swimming period, and a third and final sample was collected 10 min. after the subject had left the tank. All samples were taken at once to the bacteriological laboratory for immediate examination.

Each subject 'swam' a total of ten times, and thus a total of 150 samples were collected and studied.

Bacteriological procedures

All samples were examined for the presence of coliform organisms, faecal streptococci, buccal streptococci, staphylococci, and for total plate counts.

Coliform organisms and faecal streptococci. Both the multiple tube and the membrane filter techniques were used (*Standard Methods for the Examination of Water and Wastewater*, Tenth Edition). The multiple tube test consisted of 3–10 ml., 3–1·0 ml., and 3–0·1 ml. portions of water. All positive presumptive tests were carried through the completed test and the results expressed as Most Probable Numbers (M.P.N.) per 100 ml. of sample. The membrane filter method permitted the filtration of 100 and 10 ml. portions of water, and M-Endo's medium* was used in the detection of coliform organisms and M-enterococcus medium* for isolating the faecal streptococci. All typical colonies were subcultured for complete identification.

Buccal streptococci. Attempts to determine the presence of buccal streptococci were made by passing 100 and 10 ml. portions of water through membrane filters and incubating on 'Mitis-Salivarius' agar.*

* Baltimore Biological Laboratories, Baltimore, Maryland.

Staphylococci. 10·0 and 1·0 ml. portions of sample were diluted to 100 ml. with sterile phosphate buffer and then passed through membrane filters followed by incubation on Staphylococcus no. 110 agar.*

Standard plate count. Procedures outlined in *Standard Methods for the Examination of Water and Wastewater* (Tenth Edition) were followed, and 1 and 0·1 ml. portions of water were plated, using certified plate count agar.

RESULTS

Control samples

Samples were taken of the filtered water before each subject entered the tank and all bacteriological tests were carried out. No coliform organisms, faecal or buccal streptococci, or staphylococci were ever observed. The standard plate count at no time showed any countable plates. Thus, it has been assumed that although these 50 samples may not have been sterile before each test period, the water did not contain any of the organisms under study in the volumes examined.

Table 2. *Most probable number of coliform bacteria per* 100 *ml. of water sample collected immediately after and* 10 *min. after a* 15 *min. simulated swim period*

Figures in parentheses are the M.P.N. 10 min. after the swim.

	Condition of swimmer					
	Not menstruating		Menstruating			
	Shower	No shower	Shower		No shower	
Subject			Tampon	No tampon	Tampon	No tampon
A	9·2 (9·2) 9·1 (210)	0 (0) 3·6 (9·1) 120 (9·1)	64 (120)	120 (210)	0 (0) 0 (0)	64 (64)
B	5·1 (2·2) 64 (3·6)	> 2400 (2400) 240 (120) 460 (210)	3·6 (9·1)	36 (15)	9·1 (2·2) 9·1 (5·1)	64 (64)
C	16 (16) 0 (0)	120 (9·1) 0 (3·6)	0 (0)	0 (0)	9·1 (3·6) 0 (0)	0 (0) 0 (0)
D	0 (0)	0 (0) 0 (0) 0 (0)	0 (0)	0 (0)	0 (0) 0 (0)	0 (0) 0 (0)
E	9·1 (0) 0 (7·3) 0 (0)	64 (9·1) 64 (210) 9·1 (120) 43 (93) 0 (0)			240 (120) 7·3 (36)	

Test samples

Coliform determinations

The results obtained by the multiple tube test are shown in Table 2. These results show marked variation in the numbers of coliforms shed by different indivi-

* Baltimore Biological Laboratories, Baltimore, Maryland.

duals. There is no apparent evidence that any hygienic factor is involved. The results obtained using the membrane filter are given in Table 3 and confirm this observation.

Table 3. *Number of coliform colonies on membrane filter per* 100 *ml. of water sample collected immediately after and* 10 *min. after a* 15 *min. simulated swim period*

Figures in parentheses are the counts 10 min. after the swim.

	Condition of swimmer					
	Not menstruating		Menstruating			
	Shower	No shower	Shower		No Shower	
Subject			Tampon	No tampon	Tampon	No tampon
A	0 (0)	0 (0)	37 (31)	55 (60)	0 (0)	4 (50)
	6 (9)	3 (5)	—	—	0 (0)	—
		5 (5)				
B	2 (2)	80 (120)	2 (1)	5 (4)	28 (30)	34 (40)
	9 (7)	19 (17)	—	—	5 (5)	—
		25 (24)				
C	44 (60)	20 (12)	0 (0)	0 (0)	6 (2)	0 (0)
	20 (16)	3 (1)	—	—	0 (1)	20 (10)
D	0 (0)	1 (0)	0 (0)	1 (0)	0 (0)	0 (0)
		2 (0)	—	—	2 (0)	3 (5)
		4 (4)				
E	0 (1)	16 (12)	—	—	2 (0)	—
	3 (2)	90 (170)	—	—	3 (1)	—
	1 (1)	22 (10)	—	—	—	—
		1 (2)				
		140 (70)				

Faecal streptococcus determination

The results of the multiple tube test for demonstrating the presence of faecal streptococci are shown in Table 4 and of the membrane filter method in Table 5. These organisms were not isolated in as great numbers as was the case for coliforms, nor were they always present concurrently. Coliform organisms were present in 28 samples tested by the multiple tube test while faecal streptococci were isolated from only 18 of them. Using the membrane filter method, coliforms were isolated from 39 samples while faecal streptococci were obtained from only 30 of them.

Buccal streptococcus determination

The determination of buccal streptococci was carried out using only 1·0 ml. of the sample and in all probability does not represent an accurate index of the total number. On the medium used (M–S agar) many other micro-organisms grew readily. Strains of *Alcaligenes*, *Bacillus*, *Micrococcus*, *Sarcina*, and yeasts tended to overgrow *Strep. salivarius* when larger quantities of water were filtered. Typical mucoid colonies of *Strep. salivarius* were readily recognized and identified, however, from the 1·0 ml. samples, and it appears to be evident that individuals shed these organisms on many occasions (Table 6).

Table 4. *Most probable number of faecal streptococci per* 100 *ml. of water sample collected immediately after and* 10 *min. after a* 15 *min. simulated swim period*

Figures in parentheses are the M.P.N. 10 min. after the swim.

	Condition of swimmer					
			Menstruating			
	Not menstruating		Shower		No shower	
Subject	Shower	No shower	Tampon	No tampon	Tampon	No tampon
A	2·2 (5·1)	0 (9·2)	1100 (1100)	0 (240)	0 (0)	1100 (1100)
	0 (460)	0 (0)	—	—	0 (0)	—
		0 (0)				
B	16 (5·1)	—	64 (64)	64 (9·1)	16 (5·1)	120 (240)
	64 (3·6)	64 (120)	—	—	2·2 (0)	—
		64 (64)				
C	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	0 (0)	64 (0)	—	—	0 (0)	0 (0)
D	0 (0)	9·1 (3·6)	0 (0)	0 (0)	0 (0)	0 (0)
		0 (0)	—	—	0 (0)	0 (0)
		0 (0)				
E	1100 (1100)	0 (0)	—	—	0 (0)	—
	0 (0)	64 (64)	—	—	0 (0)	—
	0 (0)	0 (0)				
		0 (0)				
		0 (0)				

Table 5. *Number of faecal streptococcus colonies on membrane filter per* 100 *ml. of water sample collected immediately after and* 10 *min. after a* 15 *min. simulated swim period*

Figures in parentheses are the numbers 10 min. after the swim.

	Condition of swimmer					
			Menstruating			
	Not menstruating		Shower		No shower	
Subject	Shower	No shower	Tampon	No tampon	Tampon	No tampon
A	5 (8)	0 (0)	4 (5)	43 (66)	0 (0)	66 (68)
	0 (0)	1 (2)	—	—	0 (0)	—
		0 (0)				
B	34 (40)	38 (33)	6 (3)	6 (8)	15 (22)	32 (30)
	7 (0)	30 (16)	—	—	10 (22)	—
		70 (80)				
C	0 (0)	9 (8)	0 (0)	0 (0)	0 (0)	0 (1)
	1 (3)	36 (27)	—	—	0 (0)	0 (0)
D	4 (1)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		2 (0)	—	—	0 (0)	0 (0)
		3 (2)				
E	0 (9)	0 (0)	—	—	0 (0)	—
	7 (3)	6 (8)	—	—	0 (0)	—
	1 (1)	3 (1)				
		14 (6)				
		66 (70)				

Staphylococcus detection

Not all the samples collected were studied for the presence of staphylococci. However, all ten samples involving subject E, the last seven involving subject D, and the last six involving subjects A, B, and C were examined for both the presence of staphylococci in general and *Staph. aureus* in particular. The results are given in Table 7, and it is evident that members of this genus were consistently shed in relatively large numbers by all subjects under all conditions. *Staph. aureus* was

Table 6. *Number of* Streptococcus salivarius *colonies on membrane filter per* 1·0 *ml. of water sample collected immediately after and* 10 *min. after a* 15 *min. simulated swim period*

Figures in parentheses are the counts 10 min. after the swim.

	Condition of swimmer					
			Menstruating			
	Not menstruating		Shower		No shower	
Subject	Shower	No shower	Tampon	No tampon	Tampon	No tampon
A	0 (0)	0 (0)	0 (0)	10 (23)	4 (3)	0 (0)
	0 (0)	14 (15)	—	—	0 (0)	—
		0 (1)				
B	8 (6)	0 (9)	0 (0)	8 (15)	0 (0)	8 (9)
	0 (0)	2 (5)	—	—	0 (0)	—
		0 (0)				
C	1 (2)	1 (1)	0 (0)	1 (0)	2 (1)	0 (0)
	0 (0)	1 (2)	—	—	0 (0)	0 (0)
D	3 (1)	36 (7)	0 (0)	0 (0)	0 (0)	1 (0)
		0 (0)	—	—	0 (0)	8 (15)
		0 (0)				
E	15 (2)	20 (10)	—	—	1 (0)	—
	0 (0)	4 (4)	—	—	9 (0)	—
	10 (5)	2 (2)				
		24 (30)				
		10 (8)				

present in water collected during each experiment in varying numbers, although it was not present in all the samples examined. There did appear to be fewer staphylococci shed by each individual after a shower than when no shower was taken and further study is indicated.

Total plate counts

The results of the standard plate count are shown in Table 8. They indicate only that a relatively large number of micro-organisms are shed into the water at all times and that they persist after the swimmer leaves the tank. No attempt was made to identify any of the bacterial colonies.

Table 7. *Numbers of staphylococcus and* Staphylococcus aureus *colonies on membrane filter per* 1·0 *ml. of water sample after a* 15 *min. simulated swim period*

Subject	Condition of swimmer: Not menstruating — Shower		Not menstruating — No shower		Menstruating — Shower — Tampon		Menstruating — Shower — No tampon		Menstruating — No shower — Tampon		Menstruating — No shower — No tampon	
	A. *Sample collected immediately after the swim period*											
A	61	**0**	35	**2**	96	**16**	47	**11**	—	—	130	**30**
			180	**8**								
B	34	**6**	24	**16**	50	**1**	200	**6**	—	—	80	**13**
			300	**42**								
C	42	**0**	77	**0**	48	**0**	42	**5**	58	**2**	37	**2**
D	11	**0**	78	**4**	—	—	—	—	76	**2**	120	**0**
			86	**0**							200	**0**
			23	**0**								
E	TNC	**0**	TNC	**TNC**	—	—	—	—	56	**7**	—	—
	52	**5**	350	**18**	—	—	—	—	260	**200**	—	—
	19	**5**	180	**20**								
			34	**0**								
			250	**10**								
	B. *Sample collected* 10 *min. after the swim period*											
A	48	**8**	33	**0**	48	**6**	42	**8**	—	—	140	**34**
			180	**5**								
B	13	**0**	21	**1**	51	**0**	220	**7**	—	—	79	**9**
			280	**35**								
C	43	**2**	54	**3**	35	**4**	5	**0**	60	**1**	26	**0**
D	12	**3**	120	**4**	—	—	—	—	89	**4**	91	**2**
			92	**1**	—	—	—	—	—	—	250	**15**
			18	**1**								
E	62	**5**	TNC	**TNC**	—	—	—	—	34	**6**	—	—
	42	**3**	370	**27**	—	—	—	—	230	**180**	—	—
	22	**2**	170	**10**								
			30	**7**								
			200	**5**								

The figures in bold type are counts of *Staph. aureus*.
TNC = Too numerous to count.

DISCUSSION

Since this preliminary investigation involved only a limited number of observations for each category of hygienic conditions, discussion must be limited to generalizations.

Occurrence of faecal organisms

The five participants were healthy, active young women with excellent hygienic habits; yet a marked variation in the numbers of faecal micro-organisms shed into the test water occurred under identical circumstances. Subjects B and D represent the two extremes, the former constantly shedding both coliforms and faecal streptococci under all conditions and the latter showing only minimal counts and

then only when 100 ml. portions of water were examined by the membrane filter procedure.

The subjects showing the lowest incidence of faecal organisms were the ones who regularly used a soap containing hexachlorophene. The significance, if any, of this is not apparent at present and further investigations of this point are indicated.

Swimming while mentruating did not appear to affect the number of faecal organisms washed into the water.

Table 8. *Standard plate counts per* 1·0 *ml. of sample collected immediately after and* 10 *min. after a* 15 *min. simulated swim period*

Figures in parentheses are the counts 10 min. after the swim.

Subject	Condition of swimmer											
	Not menstruating				Menstruating							
					Shower				No shower			
	Shower		No shower		Tampon		No tampon		Tampon		No tampon	
A	14	(11)	4	(2)	93	(110)	100	(65)	0	(8)	20	(31)
	1300	(1200)	240	(240)	—		—		—	—	180	(180)
			650	(800)								
B	18	(25)	11	(18)	110	(130)	340	(600)	210	(250)	410	(330)
	270	(430)	500	(560)	—	—	—	—	590	(620)	—	—
			920	(900)								
C	45	(160)	710	(810)	32	(22)	8	(240)	14	(94)	470	(770)
	240	(70)	68	(100)	—	—	—	—	36	(46)	1500	(940)
D	65	(95)	330	(320)	750	(390)	1300	(1100)	1300	(1800)	34	(680)
			760	(440)	—	—	—	—	1800	(440)	1800	(440)
			4	(5)								
E	1000	(820)	1600	(3600)	—	—	—	—	720	(900)	—	—
	350	(350)	450	(310)	—	—	—	—	1200	(1100)	—	—
	76	(170)	290	(280)								
			20	(54)								
			560	(460)								

A comparison of the multiple tube method and the membrane filter procedure leads to the conclusion that the latter is to be preferred for the following reasons: (1) varying amounts of the sample under test may be used, thus permitting evaluation of organisms present in low concentrations; (2) results are obtained within 12–18 hr; (3) isolated colonies may be selected easily for further identification; (4) the method can be adapted, if desired, for use at the source of the sample, thus eliminating variables introduced by transport to the laboratory.

The choice between coliform organisms and faecal streptococci as indices of pollution in swimming pool waters may be evaluated on the basis of the present study. Using the multiple tube test, 19 samples were negative for both groups, 18 samples were positive for both groups, 11 samples were found to have coliforms present but no faecal streptococci, and only 2 samples were positive for the latter and negative for the former. With the membrane filter method, 9 samples were negative for both groups, 28 samples were positive for both groups, 11 samples

were found to have coliforms but no faecal streptococci, and only 2 samples were positive for faecal streptococci and negative for coliforms. Thus, it is apparent that coliform bacteria represent more frequently the presence of contamination from swimmers than do the faecal streptococci.

The fact that the majority of the subjects contributed these enteric organisms to the test water lends merit to their continued use as indicators of pollution. Furthermore, the ability of these organisms to persist in the water for 10 min. after the swimmer had left the tank seems to emphasize the need for continuous residual chemical disinfection of swimming pool water.

Occurrence of body cocci

The results clearly show that the greatest number of micro-organisms shed into bathing waters by the subjects under study were cocci. Representative isolates of colonial types from the M–S agar indicated that members of the genera *Neisseria*, *Sarcina*, *Micrococcus*, and *Staphylococcus* were present. All colonies resembling *Staph. aureus* were isolated from the Staphylococcus no. 110 agar and were so classified if found to be deep yellow- or orange-pigmented, coagulase-positive, mannitol-fermenting, Gram-positive cocci. These organisms were found to be present throughout the test although they were not isolated from every water sample. However, it is clear that at all times and under all conditions of swimming, *Staph. aureus* may be shed into the water. Since generally there were lower counts of both total staphylococci and *Staph. aureus* following a shower, there is presumptive evidence, at this point, that there is some merit in the requirement for showering with warm water and soap before entering a swimming pool.

Subjects B and E, neither of whom regularly used a soap containing hexachlorophene, generally showed higher counts for these organisms than the other subjects, but it should be emphasized that this should be further explored before definite conclusions are drawn.

It is evident that body cocci are omnipresent, and clearly shown that these organisms must be considered in determining procedures for disinfecting swimming pool waters.

Streptococcus salivarius has been suggested by certain workers as an index of pollution. This organism, however, was recovered from only 28 of the 50 samples examined and thus it does not appear to be as good an index as coliform bacteria. Furthermore, this organism requires a special culture medium for isolation which, although specifically designed to encourage its growth, proved to be an excellent medium for a number of other organisms, including members of the genera *Micrococcus*, *Sarcina*, *Staphylococcus*, *Streptococcus*, *Alcaligenes*, *Bacillus*, *Lactobacillus*, and unidentified yeasts.

SUMMARY

Five healthy young women swam in untreated water of known bacterial quality under a variety of hygienic conditions. Evidence based on bacteriological examination of water samples leads to the following conclusions:

1. There is a marked variation in the number and types of bacteria shed by a

bather while swimming and the variations do not seem to be correlated to the differences in personal hygiene or the menstrual period.

2. Faecal organisms may be discharged in considerable numbers by a swimmer after a thorough and careful shower with soap and warm water and yet not be discharged in appreciable numbers by a bather who does not take a shower before swimming.

3. Faecal organisms constitute only a small minority of the total number of viable bacteria that are discharged in swimming pool water by a bather during the act of swimming and as such seem to have limited use as indicators of *total* bacterial pollution.

4. Members of the genus *Staphylococcus* are shed in large numbers under all conditions and *Staph. aureus* is consistently present. Therefore, this genus appears to be a good choice as an index for the determination of body contamination.

5. Further studies are indicated under more stringently controlled hygienic conditions to determine the value of hexachlorophene in reducing microbial flora that a given individual may shed during swimming.

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Acquisition of *Staphylococcus aureus* by patients undergoing cytotoxic therapy in an ultra-clean isolation unit

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The purpose of this paper is to report the results of bacteriological studies carried out in a special unit. The unit was designed for the intensive treatment of patients by cytotoxic drugs in a clean environment and a general description of it has been presented elsewhere (Bagshawe, 1964). It is generally accepted that patients receiving these drugs have an increased susceptibility to infection and where repetitive exposure to these drugs is necessary the risks are substantial. The majority of patients with post-gestational choriocarcinoma can be cured by cytotoxic treatment which lasts three to 18 months (Bagshawe, 1963; Ross *et al.* 1965), and it seems desirable that mortality from infection during the treatment of a potentially curable disease of young adults should be avoided. In view of the long period of treatment no attempt was made to achieve bacteriological sterility of the patients under study. The unit provides an opportunity to define and analyse the problems in reducing risk of infection by exclusion of pathogens and by monitoring the endogenous flora under conditions which are acceptable for long periods by both patients and nursing staff. The data presented here relate to nasal carriage, acquisition of and infection by *Staphylococcus aureus* (= coagulase positive staphylococci), in the twenty-five patients admitted to the unit during the year following its opening in March 1964.

THE PATIENTS

Source of patients, diagnoses, age, sex and previous therapy

Six patients were admitted direct to the unit from referring hospitals and nineteen were admitted after a period of investigation and sometimes after cytotoxic treatment in the open medical wards at Fulham Hospital. The patients included one man aged 52 years with an anaplastic carcinoma from an unidentified primary site. The others were women aged 18–35 years with trophoblastic tumours. One patient had an ovarian non-gestational choriocarcinoma and the remaining twenty-three patients had either post-gestational choriocarcinoma or invasive mole (chorioadenoma destruens). Eight patients were known to have received antibiotic therapy shortly before admission to the unit and eighteen had received cytotoxic therapy before admission.

Cytotoxic therapy

All the patients received courses of methotrexate usually in combination with 6-mercaptopurine and some also received actinomycin D and alkylating agents. Courses of treatment generally lasted 3–10 days and produced moderate to severe toxic symptoms with stomatitis, skin eruptions, hair loss, etc. The intervals between courses of treatment were generally 7–14 days. Courses were administered repetitively until there was no clinical or hormonal evidence of residual disease. Eight patients received cytotoxic therapy by continuous infusion into the low aorta. Infusion catheters introduced by the Seldinger technique (Seldinger, 1953) were *in situ* for periods of 4–12 weeks.

The male patient and the patient with ovarian choriocarcinoma died from progressive disease during the period of study.

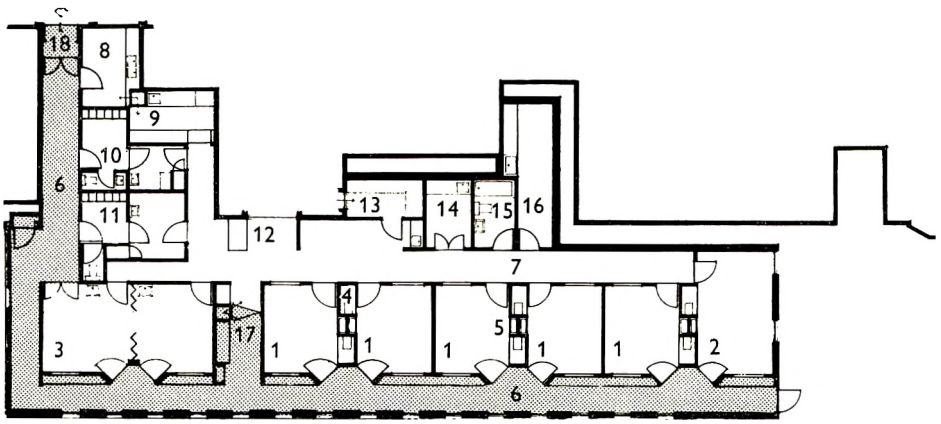

Fig. 1. Reproduced by kind permission of the *British Medical Journal*. Layout of unit. (1) Standard room. (2) Patient's room or sitting room. (3) Special procedure room or two separate rooms. (4) Lavatory. (5) Recessed wash-bowl. Television above. (6) Service and visitors' corridor. (7) Nursing area. (8) Wash-up kitchen. (9) Food-preparation kitchen. (10) Men's changing rooms. (11) Women's changing rooms. (12) Sister's desk. (13) Delivery hatch into linen store. (14) Sterilizing room. (15) Bathroom. (16) Store room fitted with washing and drying machine. (17) Sluice fitted with bed-pan washer and sterilizer accessible both from dirty and clean sides. (18) Entrance from main hospital corridor. (Architects: Gore, Gibberd, and Saunders; Consulting Engineers: Donald, Smith, Seymour, and Rooley; Main Contractors: Marshall Andrew and Co. Ltd.)

THE UNIT

Layout of the unit

The unit consists of eight patient rooms and during the period of the present study six of these were in use in the manner described here (Fig. 1).

One room was used for two patients throughout. Access to the patients' room is from the clean corridor side only and the clean corridor can be entered only through the changing rooms. Visitors can see and talk to patients through a transparent panel, but do not enter the unit. The only persons admitted to the unit are patients, ward staff and personnel required to service equipment within the unit. The unit was designed to reduce the need to admit non-medical staff to a minimum.

Air conditioning

Two-stage microcell filters remove particles larger than 0·5 μ. The air temperature is $25 \pm 1{\cdot}5°$ C., humidity 65 %, with eight changes of air per hour in the patients' rooms, supplied by inlet ports in the ceilings. Fifty per cent of the input is extracted at floor level in the patients' rooms and at ceiling level in the lavatory; the other 50 % maintains the gradient and leaks away. The patients' rooms are positive with respect to the nursing area and the nursing area is positive with respect to the exterior. Recirculation of the air is not employed.

ROUTINE PROCEDURES

General

One per cent Tego (an ampholytic quaternary ammonium compound) is used to wash the floors, walls and all hardware daily, and also for washable foodstuffs which are subsequently rinsed in tap water. Cooked foods obtained from the hospital diet kitchen are reheated in a microwave oven, (Lacey *et al.* 1965).

Letters, magazines, papers and other things not damaged by heat were sterilized in the hot air oven at 65° C. for $1\frac{1}{2}$ hr. Goods from the Central Sterile Supply were double wrapped and the outer wrappings were removed at the entry port. Mattresses, pillows and blankets (cotton) were sterilized periodically by gamma irradiation. Other bed linen and laundry were autoclaved. Patients' and staff underwear were washed in a Bendix washer-dryer unit located inside the unit. Bedpans, crockery and all goods used in the patients' rooms were removed through two-way cupboards to the service corridor. Bedpans were washed in a standard washer, passed into a Dishlex bedpan sterilizer from the service corridor side and removed from the clean corridor side. Crockery was returned to the outer kitchen, and passed into a Westinghouse dishwasher (drying temperature 96° C.). Ophthalmoscopes, etc., were wiped with 1 % Tego after use.

Staff

Staff stripped completely on each admission to the ward, washed hands and face with Cidal soap (Hexachlorophane 1 %), and put on clean uniform (this includes nylon tights for the nursing staff). Cidal soap was provided for use on duty and at home. Hexachlorophane coal tar shampoo was supplied for use weekly. Nose, throat and hand swabs were cultured weekly on blood agar. Selective media were not used. Sensitivity testing was done with an Oxoid multodisk containing penicillin 5 units, tetracycline 50 μg., streptomycin 25 μg., erythromycin 50 μg.; chloramphenicol 50 μg. Naseptin nasal cream (chlorhexidine hydrochloride 0·1 %, neomycin sulphate 0·5 %) was applied to the anterior nares for 1 week after a positive nasal swab, or until the next negative weekly swab. In addition to medical and nursing staff in the unit the study includes orderlies and cleaners employed in the wash-up room and service corridor.

Patients

All patients were bathed on admission, in the bathroom if mobile, blanket-bathed if not. Hair was washed on admission and at least once weekly with hexachlorophane-coal tar shampoo. Hands were washed with Cidal soap only. Patients were graded according to total WBC count, done three or more times weekly.

(A) WBC > 2000/mm.^{3}	Patient could use bathroom, i.e. leave own room, but could not enter any other patient's room. They could talk to one another in the corridor
(B) WBC 2000-1000/mm.^{3}	Patient confined to room. Staff wear masks and wash hands immediately before entering
(C) WBC < 1000/mm.^{3}	As B but staff put on sterile gown before entering

Swabs from the nose, throat and rectum (if stool not available), and mid-stream urine specimens were collected weekly. Patients with arterial catheters had weekly swabs taken from the entry site. Patients with *Staph. aureus* on nose swabs were treated as described above for staff.

When febrile reactions developed in these patients, during or after a course of cytotoxic therapy, it was rarely possible to identify a causal organism. Nevertheless, when the patients were leucopenic, systemic antibiotics were usually given even though it was recognized that the reactions were not necessarily bacterial in origin. The sensitivities of the organisms recovered on the routine swabs determined the choice of antibiotics used.

RESULTS

One of six patients admitted to the unit directly from other hospitals carried a multiple resistant *Staph. aureus*. Eight of nineteen patients admitted from the general medical wards at Fulham Hospital had *Staph. aureus* on initial nasal culture and four of these were multiple resistant. All nineteen had received cytotoxic therapy in the general wards, whereas those admitted directly from other hospitals had not.

Table 1 shows the nasal carrier rate of *Staph. aureus* of patients in the unit. The initial swabs were collected during the week preceding admission (from patients transferred from the medical wards) or within 24 hr. after admission to the unit. The number of initial swabs is small, but there does appear to be a reduction of nasal carriage of *Staph. aureus* after admission to the unit. The figures for nasal carriage by the staff of the unit are shown in Table 2. Comparison with an obstetric ward in the same hospital group (Table 3) demonstrates a lower total nasal carriage by the unit staff (15·6 % against 24·3 % by the obstetrics staff) accounted for mainly by the differences in carriage of the *Staph. aureus* resistant to penicillin only. The patients of both wards show a similar total nasal carriage rate, but whereas three quarters of the unit patients' *Staph. aureus* were multiple resistant, only one-sixth of those of the obstetrics patients were multiple resistant.

Antibiotic treated unit patients carried a higher proportion of multiple resistant *Staph. aureus* and a smaller proportion resistant to penicillin only, although the total carriage rates were similar (Table 4). The number of patients who did not

receive any antibiotic is small and the difference between the treated and untreated groups is not significant.

The rate of total and of multiple resistant *Staph. aureus* nasal carriage by patients was four times as high in those who had several WBC counts of less than 1000/mm.^{3} than in those who had not more than one count of less than 1000/mm.^{3} This difference was highly significant ($\chi^2 = 12{\cdot}2$, $P = < 0{\cdot}01$) (Table 5).

Table 1. *Nasal carriage of* Staph. aureus *by patients on admission to the unit compared with the results of subsequent weekly nose swabs*

	Initial		Weekly	
Total swabs cultured ...	25		367	
Staph. aureus	No.	%	No.	%
Sens.	1	4	4	1·1
R/P	3	12	9	2·5
R/T	—	—	1	0·3
R/M	5	20	53	14·5
Not known	—	—	2	0·5
Total	9	36	69	18·8

Notes:
Sens. = sensitive to penicillin, tetracycline, streptomycin, erythromycin and chloromycetin.
R/P = resistant only to penicillin.
R/T = resistant only to tetracycline.
R/M = resistant to two or more antibiotics, usually penicillin and tetracycline.

Table 2. *Nasal carriage of* Staph. aureus *by isolation unit staff*

	April 1964–March 1965–	
Total swabs cultured ...	786	
Staph. aureus	No.	%
Sens.	54	6·9
R/P	24	3·0
R/T	4	0·5
R/M	40	5·1
Total	122	15·6

Notes as for Table 1.

Table 3. *Nasal carriage of* Staph. aureus *by staff and patients on an obstetric ward in the same group*

	Patients		Staff	
Total swabs cultured ...	465		542	
Staph. aureus	No.	%	No.	%
Sens.	26	5·6	18	3·3
R/P	45	9·7	88	16·2
R/T	—	—	2	0·4
R/M	14	3·0	24	4·4
Total	85	18·3	132	24·3

Notes as for Table 1.

Table 4. *Nasal carriage of* Staph. aureus *by patients—effect of antibiotic therapy*

	Patients who received no antibiotics (6 patients)		Patients treated with antibiotics (19 patients)	
Total swabs ...	40		327	
Staph. aureus	No.	%	No.	%
Sens.	0	—	4	1·2
R/P	4	10·0	5	1·5
R/T	—	—	1	0·3
R/M	3	7·5	50	15·3
Not known	—	—	2	0·6
Total	7	17·5	62	18·9

Notes as for Table 1.

Table 5. *Nasal carriage of* staph. aureus *by patients whose WBC count fell below* 1000/mm.^{3} *only once or less compared with those in whom the WBC were below* 1000/mm.^{3} *on three or more occasions*

	Patients with total WBC < 1000/mm.^{3} once or less		Patients with total WBC < 1000/mm.^{3} three or more times	
Total swabs cultured ...	87		280	
Staph. aureus	No.	%	No.	%
Sens.	1	1·1	3	1·1
R/P	0	—	9	3·2
R/T	0	—	1	0·3
R/M	4	4·6	49	17·5
Not known	—	—	2	0·7
Total	5	5·7	64	22·8

$\chi^2 = 12{\cdot}2, \quad P = < 0{\cdot}01.$

Notes as for Table 1.

Table 6. *Rate of nasal acquisition of new strains of* Staph. aureus. *by patients showing the fall in rate of apparent acquisition of new strains with increasing time after admission*

	Overall	Excluding 1st 2/52	Excluding 1st 4/52	Excluding 1st 6/52
Total new strains/ patient weeks at risk	23/366	17/317	14/270	11/233
New strains/100 patient weeks	6·3	5·4	5·2	4·7
R/M new strains/ patient weeks	18/366	15/317	12/270	9/233
R/M new strains/ 100 patient weeks	4·9	4·7	4·4	3·9

Notes as for Table 1.

Following Parker, John, Emond & Machacek (1965) we considered as members of a single strain those organisms which had phage-typing patterns differing by one strong lytic reaction or less, and with the same sensitivity to penicillin and tetracycline. The rate of acquisition of strains of staphylococci new to the patient decreased progressively during the first 6 weeks in the unit (Table 6).

Table 7. *Source of apparent acquisition of new strains of nasal* Staph. aureus *by patients*

Staph. aureus	Total	Staff	Other patients	Not known
Sens.	4	2	0	2
R/P	2	0	1	1
R/M	17	7	4	6
Total	23	9	5	9

Notes as for Table 1.

Table 8. *The effect of antibiotic therapy on the patients' rate of apparent nasal acquisition of new strains of* Staph. aureus

	No antibiotics		Antibiotics	
Staph. aureus	Total	R/M	Total	R/M
Acquisitions	3	2	20	16
Weeks at risk	40		327	
Aquisitions/100 patient weeks	7·5	5·0	6·1	4·9

Notes as for Table 1.

Table 9. *The relation between leucopenia and the rate of patients' nasal acquisition of new strains of* Staph. aureus: *the rate for patients whose WBC fell below* 1000/mm.^{3} *once or less is compared with those whose WBC fell below* 1000/*mm.*^{3} *three or more times*

	Patients with leucopenia < 1000/mm.^{3} once or less		Patients with leucopenia < 1000/mm.^{3} three or more times	
Staph. aureus	Total	R/M	Total	R/M
Acquisitions	4	3	19	15
Weeks at risk	87		280	
Acquisitions/100 patient weeks	4·6	3·5	6·7	5·3

Notes as for Table 1.

The source of these apparent acquisitions was sought among the results of all swabs from staff and patients during the 6 weeks preceding acquisition (Table 7). No likely source was discovered for nine of the twenty-three acquisitions, a further nine were isolated from staff, and five from other patients. There was no difference in the number of acquisitions/100 patient weeks between patients treated or not with antibiotics, but only six patients received no antibiotics, remaining in the unit for a total of 40 weeks (Table 8).

The occurrence of several episodes of leucopenia increased the rate of apparent acquisition of new strains of nasal staphylococci, but the patients whose WBC fell below 1000/mm.^{3} once or less only totalled 87 weeks in the unit and the results are not significant at the 5% level (Table 9). Further data are being collected.

During the year under review staphylococci were recovered on twelve occasions from sites of minor infection. Seven of these positive swabs were obtained from superficial skin lesions; one from the mouth, two from conjunctivae and on two occasions from the entry points of arterial catheters in the groin. This gave an overall infection rate of 3·3/100 patient weeks.

Table 10. *Probable source of* Staph. aureus *causing patients' lesions*

Staph. aureus	Total	Staff	Other patients	Not known	Patient herself
Sens.	3	1	0	1	1
R/P	1	0	0	0	1
R/M	8	1	2	1	4
Total	12	2	2	2	6

Notes as for Table 1.

Table 11. *The effect of leucopenia on the occurrence of staphylococcal lesions: patients whose WBC fell below* 1000/*mm.*^{3} *once or less are compared with those whose WBC fell below* 1000/*mm.*^{3} 3 *or more times*

	Patients with leucopenia < 1000/mm.^{3} once or less	Patients with leucopenia < 1000/mm.^{3} three or more times
Staphylococcal lesions	1	11
Weeks at risk	87	280
Lesions/100 patient weeks	1·1	4·1

It was found that in six instances the source of the *Staph. aureus* was staff or another patient or was unknown (Table 10) and in six instances the patient's nose had been colonized before the lesion occurred. Where the patient was the direct source of the organisms in a lesion the organisms had apparently been acquired from staff and from other patients in equal numbers.

Patients who had several leucopenic episodes had nearly four times as many staphylococcal lesions/100 patient weeks as those with one leucopenic episode or less (Table 11).

Two strains of *Staph. aureus* caused more than one lesion. Five lesions in three patients were caused by type 52/52A/80/81 multiple resistant *Staph. aureus* over a period of 45 weeks. Two patients shared the double room and the third patient was in the adjacent room. Two lesions in two patients were caused by an untypable strain, which was sensitive to antibiotics tested. The patients were in adjacent rooms but the second lesion occurred 13 weeks after the first. During this interval the organism was recovered in sequence from a staff throat swab, a staff hand swab, the patient's nose, and another patient's nose swab.

DISCUSSION

Various factors are known to influence the staphylococcal carriage rate. Females aged 20–40 years were found to be least susceptible in the P.H.L.S. Report (1965). It is well known that in hospital there is a steady increase in the general carrier rate of *Staph. aureus* and in the carrier rate of resistant strains. Williams *et al.* (1959) found that the general carrier rate rose from 38% in the first week to 70% after 8 weeks, and the penicillin resistant carrier rate from 13 to 50%. The P.H.L.S. Report (1965) showed that the incidence of staphylococcal infection in patients with malignant disease was twice the overall rate for hospital patients. The incidence is also increased by corticosteroids and by antibiotics (P.H.L.S. Report, 1965). The apparent acquisition rate of new multiple resistant strains was higher in antibiotic treated patients than in those receiving no antibiotics (Parker *et al.* 1965; Knight & Holzer, 1954).

On the grounds of age and sex the patients in the present report might have been expected to have a low incidence of staphylococcal infection. Despite this and the extensive precautionary measures taken, staphylococcal acquisition occurred within the unit and there were minor instances of infection. It is clear that the underlying malignant disease may be a predisposing factor as is the use of antibiotics for febrile reactions of uncertain aetiology. Corticosteroids could not be incriminated since only one patient received these during the period under study. Surgical procedures on patients in the unit included hysterectomy (two cases), oversewing of vaginal mucosa on account of haemorrhage (four cases), and venous (approx. 140) and arterial cannulation (8 cases), but no great responsibility could be attached to these as a source of infection although *Staph. aureus* was grown from routine swabs taken from the entry point of arterial catheters in the groin in two of eight cases. These arterial catheters were however *in situ* for periods of up to 3 months and were not comparable to the cannulation reported in the P.H.L.S. survey (P.H.L.S. Report, 1965), where a sepsis rate of 2·7% was found.

The results obtained here suggest that cytotoxic chemotherapy increases susceptibility to the acquisition and nasal carriage of *Staph. aureus*. Patients admitted to the unit from other hospitals, who had not received cytotoxic drugs, had an average rate of nasal carriage for hospital patients (1/6), whereas those admitted via the medical wards at Fulham Hospital where they received cytotoxic chemotherapy had a higher carriage rate (8/19). Similarly, it was found that the nasal carriage rate was significantly higher for those patients whose treatment was more vigorous as reflected in the number of leucopenic episodes which they incurred. The acquisition of new strains by the patients with more leucopenic episodes was also higher but the difference was not statistically significant. The population studied here thus has heightened susceptibility to infection with *Staph. aureus*, and remains infected longer.

The way in which cytotoxic agents increase susceptibility to acquisition is not known but possible mechanisms can be suggested. The agents used here have gross effects on the integrity of mucosal surfaces. They are known to exert immunosuppressive effects in the dosages used, and to cause profound leucopenia.

In a study of experimental staphylococcal nasal colonization Ehrenkrantz (1966) found that 'resistance to intra nasal implantation was characterized by induction, anamnestic response and specificity—the cardinal signs of an immune reaction'.

Nasal secretions do contain immunoglobulins in the γ_1A and γ_2-globulin fractions (Remington, Vosti, Lietze & Zimmerman, 1964; Bellanti, Artenstein & Buescher, 1965) but a clear relationship between serum antibodies and rejection of nasal staphylococci has not been established.

It seems likely that some acquisitions of *Staph. aureus* are apparent rather than real as suggested by Parker *et al.* (1965). That is, the organisms were present on admission but were only revealed by repeated swabbing during the first weeks after admission. The 'acquisition' rate measured in this unit fell progressively during the six weeks after admission.

Contact between patients in the unit leads to some cross-transmission of organisms. However desirable it might be to prevent this, compromise has to be made in the interests of the patients' acceptance of prolonged and often unpleasant treatment. Also, by permitting contact during periods of well-being strict isolation is more acceptable when necessary.

It is also clear from our results that the ward staff are responsible for introducing organisms which are acquired by and infect the patients. 'Nasal carriers' cannot be usefully defined since most of the staff had positive swabs at some time. Clearly, it is not practical to exclude staff carrying *Staph. aureus* from the ward. In trying to determine the effectiveness of topical anti-bacterial agents it is necessary to distinguish between the efficiency of the agent as applied according to the instruction given and the disciplinary aspect which determines whether the instructions are carried out. The carrier rate for the unit staff was lower (15·6 %) than that on the obstetric ward studied (24 %). Nevertheless, it is clear that there is room for improvement. In practice it is relatively easy to ensure that medical and nursing staff follow the instructions given but more difficult in the case of untrained personnel. Included in the present ward staff were domestics employed in the wash-up kitchen and service corridor and who assisted with ward cleaning, and these showed a particularly high carriage rate. This emphasizes the need for a high standard of discipline.

It is hoped that by defining the routes and factors involved in the access of pathogens to these patients, control measures can be progressively improved without producing less acceptable conditions. Alternative topical agents are now being used on a regular routine basis, whether or not the previous nasal swab was positive.

SUMMARY

The twenty-four young women and one man treated in an ultra-clean isolation ward should have had a low incidence of staphylococcal infection on grounds of age, sex and clean environment alone. However, they apparently acquired new strains of *Staph. aureus* at the rate of 4·7/100 patient weeks (3·9 multiple resistant strains/100 patient weeks) from the sixth week after admission onwards.

Environmental factors contributing to infection included introduction of re-

sistant strains by the patients on admission, contact between patients in the unit, and failure to eliminate nasal carriage in staff and patients.

Host susceptibility was increased by malignancy, and by antibiotic and cytotoxic therapy. The nasal carriage rate of *Staph. aureus* was significantly greater for patients with repeated episodes of leucopenia induced by cytotoxic drugs.

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Immunogenicity of experimental trachoma vaccines in baboons

I. Experimental methods, and preliminary tests with vaccines prepared in chick embryos and in HeLa cells

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INTRODUCTION

Trachoma is a form of chronic follicular conjunctivitis, usually associated with corneal lesions, and with a tendency to cicatrization in the later stages. Untreated, it is liable to impair vision, and is responsible for a vast amount of ophthalmic disability, mainly in tropical and sub-tropical countries. The causal agent is a member of the psittacosis–lymphogranuloma group of obligate intracellular micro-organisms; it was first isolated with certainty by T'ang, Chang, Huang & Wang (1957), who inoculated conjunctival scrapings into the chick embryo yolk sac. It is closely similar to—if not identical with—the agent causing inclusion conjunctivitis and inclusion blennorrhoea, first isolated by Jones, Collier & Smith (1959). For convenience, the trachoma/inclusion conjunctivitis micro-organisms are jointly referred to as TRIC agents (Gear, Gordon, Jones & Bell, 1963) and the strain designations used in this paper follow the system suggested by these authors and by Collier (1963).

Although the earlier stages of trachoma can be treated effectively with sulphonamides and some antibiotics, notably the tetracyclines, the difficulty of applying mass therapy to primitive communities for long periods prompted research into the possibility of developing a preventive vaccine. Collier (1961) showed that baboons are susceptible to conjunctival infection by the MRC–4 strain of inclusion conjunctivitis (formerly known as LB4); and that two spaced subcutaneous doses followed by an intravenous injection of a live vaccine prepared from this strain conferred virtually complete protection against challenge by the conjunctival route. Replacement of the final intravenous dose by a third subcutaneous injection conferred partial immunity.

The researches described here are an extension of this work, and are part of a collaborative investigation of trachoma vaccine by the Medical Research Council's Trachoma Research Unit, Pfizer Ltd., Evans Medical Ltd., and the Lister Institute of Preventive Medicine, under the auspices of the National Research Development Corporation.

The first two papers in this series deal with experimental methods, and with studies by the M.R.C. Trachoma Research Unit on live vaccines prepared in

chick embryos or in HeLa cells; the third describes collaborative experiments by the Unit and by Pfizer Ltd. with inactivated vaccines. The methods of challenge, clinical and microbiological examinations and scoring of results were devised by the Unit, and were identical in both laboratories.

MATERIALS AND METHODS

General plan of experiments

Groups of young baboons were inoculated with various TRIC vaccines, and challenged, usually 10 days after the final dose, by rubbing live TRIC agent into the upper and lower conjunctival sacs of one eye (Collier, 1961). The course of the subsequent infection was compared with that in control animals challenged similarly.

TRIC agents

The trachoma and inclusion conjunctivitis strains used are shown in Table 1. In the text, 'fast-killing' variants that kill chick embryos comparatively quickly and grow readily in cell cultures (Reeve & Taverne, 1963) are distinguished from their 'slow-killing' parent strains by the suffix *f* (Taverne, Blyth & Reeve, 1964*a*).

Table 1. *Designations of trachoma and inclusion conjunctivitis (TRIC) agents*

Full designation	Abbreviation	Reference
TRIC/ /GB/MRC–1/G (formerly LB 1)	MRC–1/G	Jones, Collier & Smith, 1959
TRIC/2/SAU/HAR–2/OT (formerly SA 2)	SAU/HAR–2	Murray *et al.* 1960
TRIC/ /WAG/MRC–221/OT	MRC–221	Collier, Sowa, Sowa & Blyth, 1963
TRIC/ /GB/MRC–4/ON (formerly LB 4)	MRC–4	Jones, 1961; Jones & Collier, 1962

It should be noted that strain MRC–4 used for the vaccine experiments was isolated from the eye of an English baby with neonatal inclusion conjunctivitis; inoculated into the eye of an adult volunteer it induced a syndrome indistinguishable from trachoma (Jones & Collier, 1962). The course of infection in the baboon conjunctiva has been described in detail (Collier, 1961, 1962).

Yolk sac vaccines

Yolk sac vaccines described in this and the subsequent two papers were suspensions of heavily infected membranes collected soon after death of the embryos. They were partially purified by centrifugation, using methods similar to those of Collier (1961), and were finally suspended in phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954) or in sucrose potassium glutamate (SPG) (Bovarnick, Miller & Snyder, 1950); 1–5 ml. of antigen were obtained from each gram (wet weight) of yolk sac. The vaccines were stored in 1 ml. amounts in ampoules at $-60°$ C. or $-70°$ C. until required.

HeLa cell vaccines

The seed material was a suspension of MRC–4 *f* that had been passaged 18 times in chick embryos, and then 3–8 times in HeLa cells.

HeLa cells were grown in Roux bottles with 50 ml. of medium (Hanks' saline containing 10% calf serum, 5% human serum, 0·5% lactalbumen hydrolysate and streptomycin 100 μg./ml.). Monolayers were seeded with a dilution of MRC–4 *f* that formed inclusions in 80–100% of cells; 42–55 hr. later the cells were suspended in their medium by shaking with glass beads, deposited by centrifugation at 8000 *g* for 20 min., and resuspended in one-eighth of the original volume with SPG containing 10% human serum. They were then treated for 5 min. with ultrasonic vibrations from a Dawe disintegrator with a power output of 60 W. at a frequency of 20 kc./sec. (Furness & Fraser, 1962). After centrifugation at 8000 *g* for 20 min., the deposits were finally suspended in SPG containing streptomycin at 500 μg/ml. All manipulations, including centrifugation and ultrasonic treatment, were done at 0–4° C. One Roux bottle yielded 0·5–1·0 ml. of vaccine; each batch was prepared on the day of use.

Challenge inocula

Inocula were crude or partially purified yolk sac suspensions in PBS or SPG, and were held at −60° C. until used.

Titrations of infectivity and lethality in chick embryos

Serial tenfold dilutions of TRIC suspensions were made in PBS or SPG, and each inoculated in 0·3 ml. amounts into the yolk sacs of at least five 7-day chick embryos. Specificity of death was confirmed by examining Giemsa stained yolk sac smears for elementary bodies. Titres were calculated by Thompson's (1947) method in terms of infectivity (EID50/ml.) or lethality (ELD50/ml.).

Infectivity titrations in HeLa cells

The method of Furness, Graham & Reeve (1960) was used; titres are expressed as inclusion forming units (IFU) per ml.

Complement fixation tests

Serum antibodies fixing complement with psittacosis–lymphogranuloma–trachoma (PLT) group antigen were titrated on Perspex plates similar to those described by Fulton & Dumbell (1949). Sera from baboons immunized with yolk-sac antigens were tested with HeLa cell antigens and vice versa.

Blood samples were taken from the femoral vein; sera were stored at 4° C. with 0·25% sodium azide as preservative. To remove anticomplementary activity, sera were diluted 1/5 on the day of test with 1/10 Richardson's preserved complement and incubated at 37° C. for 60 min.; they were then inactivated at 56° C. for 30 min.

Yolk sac group antigen. A 20% suspension of yolk sacs heavily infected with the MRC–1/G strain of TRIC agent was centrifuged at low speed to remove coarse

particles; it was then treated with an equal volume of 2M-KCl and centrifuged at 8000 *g* for 30 min. to remove adventitious protein, which was discarded with the supernatant. The deposited elementary bodies were suspended in 1 ml. calcium-magnesium saline (Reeve & Taverne, 1962) for each yolk sac used, and heated at 100° C. for 20 min. After adding sodium azide to a final concentration of 0·25%, the antigen was stored at 4° C. Control antigen was prepared in the same way from normal yolk sacs.

HeLa cell group antigen was made from cells heavily infected with the SAU/HAR–2*f* strain by a method similar to that used for HeLa cell vaccine, except that the diluent was PBS containing 5% sodium glutamate, and, after heating at 100° C. for 20 min., the final suspension was freeze-dried for storage.

Both yolk sac and HeLa cell antigens were used at optimally effective concentrations determined by titrating them against ascending dilutions of known positive sera.

Complement. Richardson's preserved complement was diluted to contain two minimal haemolytic doses (MHD) per unit volume used in the test.

Diluents. Sera were diluted in calcium–magnesium saline, and antigen and complement in calcium-magnesium saline containing 2% inactivated human serum.

Haemolytic system. Sheep red cells were thrice washed and diluted to 0·25% with 0·85% saline. They were sensitized for 5 min. at room temperature with an equal volume of haemolysin diluted to contain 10 MHD per unit volume used in the test.

Antibody titrations. The unit volume was 0·02 ml. dispensed by dropping pipettes. One vol. each of serum, complement and antigen were added in this order to the appropriate squares on the Perspex plates, which were then left at 4° C. overnight in a moisture saturated atmosphere. On the following day 2 vols. of sensitized sheep cells were added to each square, and the plates were put in the humidity box at 37° C. for 60 min. The serum titre was taken (by interpolation when necessary) as the highest dilution giving 50% lysis. Appropriate controls for all reagents were included in each test.

Baboons

Young baboons (*Papio cynocephalus*) of either sex weighing 3–4 kg. were used. All manipulations were done under general anaesthesia induced by giving 15–20 mg. phencyclidine hydrochloride ('Sernylan', Parke Davis and Co. Ltd.) intramuscularly.

Conjunctival inoculations, and examinations for inclusion bodies

The methods of Collier (1961) were used, except that checking of negative or doubtful conjunctival scrapings by a second examiner was omitted; it was found that the very small number of false negatives revealed by this check did not justify the considerable extra work involved.

Scoring system for assessing the severity of response to conjunctival challenge

The use of a scoring system for assessing the severity of conjunctival infection was suggested by Dawson, Jawetz, Thygeson & Hanna (1961). In our experiments arbitrary numerical scores, which are amenable to statistical analysis, were allotted to the various physical signs induced by the challenge. Scores for signs of inflammation and for follicular hyperplasia were recorded separately, since these lesions appear to be expressions of different pathological processes.

Signs of inflammation comprise (*a*) external oedema of the lids, (*b*) purulent discharge, (*c*) conjunctival hyperaemia and (*d*) conjunctival infiltration (recognized by loss of transparency, oedema and thickening). Each of these signs was allotted a score of 1, 2 or 3 according to severity, 0 if absent. Conjunctival hyperaemia and infiltration were scored separately for the upper and lower lids.

Follicular hyperplasia was scored separately for upper and lower lids, using the same scale as that for inflammation. Superficial translucent follicles characteristic of 'non-specific folliculosis' were sometimes present in normal animals and were ignored (Collier, 1961.)

Inclusion bodies. The presence of inclusions in whatever numbers was allotted the maximum score of 3, since their finding constitutes an important sign of the specificity of infection.

Table 2 shows how individual scores were derived, and is an example of a severe infection.

Table 2. *Example of use of scoring system for baboons infected with TRIC agents by the conjunctival route*

(This example refers to one examination of one animal.)

Physical sign		Score	
Slight external oedema	Upper lid	1	Total score for signs of inflammation = 10
Moderate discharge		2	
Severe hyperaemia		3	
Moderate infiltration		2	
Moderate hyperaemia	Lower lid	2	
No infiltration		0	
Moderate follicular hyperplasia, upper lid		2	Total score for follicular hyperplasia = 3
Slight follicular hyperplasia, lower lid		1	
Inclusion bodies present (upper and/or lower lid)			= 3
			Total score = 16

Analysis of scores

In most experiments, the animals were examined 1, 2, 3, 4 and 6 weeks after challenge. The cumulative score for each animal for all examinations up to and including the 4th week after challenge is the basis for interpreting the results, and, except when otherwise stated, comprises four successive examinations. By the 6th week spontaneous regression of physical signs diminished the differences between control and vaccinated animals, and inclusion of the scores at this final examination yielded no additional information.

For each group the square roots of the cumulative scores for each animal up to

and including the 4th week were summed and divided by the number of animals in the group to give the mean score in terms of square roots. Using the square roots of the scores, significance of differences between immunized and control animals in their response to challenge was tested by the analysis of variance. In the tables of results, the least significant difference at the level $P = 0{\cdot}05$ is given for comparison with the actual differences.

Examination of the range of the transformed scores indicated a slightly higher overall variability in the immunized groups than in the control groups, and suggested looking for individual differences in response within groups of immunized animals. This was done by making an estimate of error from all experiments and calculating the least significant difference at the $P = 0{\cdot}05$ level between the transformed score for single animals and the mean of the transformed scores for control animals in the same experiment. These differences were added and subtracted from the corresponding mean score for the control group, and the resulting limits transformed back to the original score, to give 95% confidence limits to the scores of individual animals. Thus individual scores smaller than the lower limit suggested protection by the vaccine; and those greater than the upper limit suggested an enhanced response to challenge.

DOSE OF TRIC AGENT USED FOR CHALLENGE

A strain of trachoma agent (MRC–221) and one of inclusion conjunctivitis (MRC–4) were each titrated in parallel in the baboon conjunctiva and in the chick embryo yolk sac. Both strains had been propagated exclusively in the yolk sac in which they had the growth characteristics of 'slow-killing' strains. MRC–221 was used at the 5th passage; the MRC–4 suspension was a pool of 2nd and 3rd passage membranes. Serial tenfold dilutions were each inoculated into groups of 5 or 6 chick embryos, and into 3–5 baboons. For the baboon inoculations, cotton-wool throat swabs were soaked in the diluted suspensions and rubbed across the conjunctival surfaces of the upper and lower lids of one eye, using twelve strokes for each lid. It was estimated that each animal received approximately 0·1 ml.; the infective titre of each of the undiluted suspensions was $10^{5{\cdot}0}$ EID50/ml., so that animals inoculated with a 1/10 dilution received approximately 1000 EID50.

With the higher dilutions, some animals had slight physical signs unaccompanied by formation of inclusion bodies; in others, scanty inclusions were found in the complete absence of physical signs. With MRC–4, the less dilute suspensions induced more severe infections and inclusions were more frequently found; this effect was less obvious with MRC–221. For the purpose of these experiments, induction of specific infection was assumed only when inclusions were demonstrable at some stage of infection. The numbers of animals infected with various dilutions of each strain are given in Table 3. The estimates of 50% end-points are open to the objection that the highest dilutions of both strains induced infection in one or more animals, and that the lowest dilution of MRC–221 failed to infect all the animals in the group. Assuming however that the next dilutions higher than those actually used would have failed to infect any animals, and that

undiluted MRC–221 suspension would have infected the whole group, estimates of 50% end-points (Reed & Muench, 1938) gave values of $10^{3\cdot8}$/ml. and $10^{4\cdot0}$/ml. respectively for MRC–221 and MRC–4. Thus for both strains, one baboon infectious dose (BID 50) is roughly equivalent to 10–20 EID 50. These results may be compared with those of Dawson *et al.* (1961); about 3 ELD 50 (egg lethal doses) of trachoma strain BOUR were equivalent to a minimal 'inclusion producing dose' in cynomolgus monkeys, whereas the corresponding figure for trachoma strain ASGH was more than 1000 ELD 50. Bell, Murray, Carroll & Snyder (1963) found that for the SAU/HAR–2 strain of trachoma the infectivity end-point for chick embryos approximated closely to that for the human eye.

Table 3. *Parallel titrations of TRIC agents in baboon conjunctivae and in chick embryo yolk sacs*

Strain	Dilution	No. infected / Total	Titre* BID 50/ml.	Titre EID 50/ml.	BID 50 / EID 50
MRC–221	10^{-1}	3/4	$10^{3\cdot8}$	$10^{5\cdot0}$	16
	10^{-2}	3/5			
	10^{-3}	3/5			
	10^{-4}	1/3			
MRC–4	10^{-1}	5/5	$10^{4\cdot0}$	$10^{5\cdot0}$	10
	10^{-2}	4/5			
	10^{-3}	3/5			

* Assuming 0·1 ml used to infect each animal.
BID 50 = 50% baboon infective dose.
EID 50 = 50% egg infective dose.

The findings with our strains suggest that to induce satisfactory infection with reasonable certainty in all animals of a given control group it is necessary to use an inoculum of at least 1000 EID 50, and this was done in the vaccine experiments to be described. Challenge of this order is undoubtedly more severe than anything likely to be encountered with naturally occurring infections, but the use of more dilute inocula would entail an impractically large number of baboons in order to demonstrate significant differences in behaviour between vaccinated and control groups.

IMMUNIZATION WITH LIVE AQUEOUS VACCINES

Antigen prepared in yolk sac

Experiment 2: immunization and challenge with strain MRC–4

The first experiment in this series was described by Collier (1961). Experiment 2 was also reported in part, but since the scoring system was not then in use, and additional information about the duration of immunity has since been gained, it is now given in greater detail.

The vaccine was made from yolk sacs infected with the 5th egg passage of MRC–4; its infective titre was $10^{5\cdot2}$ EID 50/ml. The vaccination schedule is given in Table 4; for subcutaneous inoculations, 0·5 ml. was injected into the thoracic aspect of each axilla, with the object of stimulating more than one group of lymph nodes.

The challenge inoculum was a partially purified pool of 2nd and 4th passage MRC–4 with an infective titre of $10^{4\cdot9}$ EID50/ml., and was inoculated into the right eyes of all animals by the conjunctival route 10 days after the final dose of vaccine.

Table 4. *Experiment 2: vaccination with aqueous suspension of live MRC–4 grown in yolk-sac: challenge with MRC–4*

No. of baboons	Day vaccinated	Dose of vaccine	Mean score ($\sqrt{}$) at 24 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D.† ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
6	0, 7, 14	1·0 ml. s.c., 1·0 ml. i.v.	1·41	−4·04	1·65	6/6
6	0, 7, 14	1·0 ml. s.c.	3·18	−2·27	1·65	3/6
6	—	No vaccine	5·45	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper = 62, lower = 9.
* That is, with individual scores of 9 or less.
† L.S.D. = least significant difference.

Response to conjunctival challenge

By way of example, individual scores for animals in one of the vaccinated groups and for the controls at each examination are given in Table 5 (but for reasons of space are not shown for subsequent experiments). Table 4 gives the transformed mean scores for vaccinated and control groups, and the least significant differences between them; it also shows the number of animals in the vaccinated groups deemed to have been protected against conjunctival challenge.

Two successive subcutaneous doses of live MRC–4 followed by an intravenous dose almost completely protected all six baboons against challenge with the homologous strain; physical signs were slight or absent, and scanty inclusions were found in only one animal 46 days after challenge (not shown in Table 5). By contrast, the unvaccinated animals all had severe or moderately severe conjunctival infection, and all were inclusion-positive at two or more examinations. Of the six baboons receiving three successive subcutaneous doses, three were protected to a significant degree.

Rechallenge after 15 months

Fifteen months after vaccination, the surviving animals were challenged in their left eyes with a pool of 3rd and 4th passage MRC–4 containing $10^{4\cdot0}$ EID50/ml.

Table 6 shows that the immunity in both vaccinated groups had by now waned considerably. Only one baboon of those receiving an intravenous dose still resisted challenge to a significant extent, even though the challenge dose was 10 times less on this occasion. One of the three animals originally protected by three subcutaneous injections died in the interim period; the remaining two failed to resist the second challenge.

Table 5. *Experiment 2: individual scores of animals immunized with two subcutaneous and one intravenous dose of MRC–4 (group A) and unvaccinated controls (group B)*

Baboon No.	Lesion	Days after challenge 0	4	9	17	24	Cumulative totals
		Group A					
29	I*	0	0	0	0	0	0
	F	0	0	0	0	0	0
	IB	0	0	0	0	0	0
	Totals	0	0	0	0	0	0
30	I	0	0	0	0	0	0
	F	0	0	0	0	0	0
	IB	0	0	0	0	0	0
	Totals	0	0	0	0	0	0
31	I	0	3	0	0	0	3
	F	0	0	0	0	1	1
	IB	0	0	0	0	0	0
	Totals	0	3	0	0	1	4
32	I	0	4	1	0	0	5
	F	0	0	2	1	0	3
	IB	0	0	0	0	0	0
	Totals	0	4	3	1	0	8
33	I	0	3	0	2	0	5
	F	0	0	2	0	0	2
	IB	0	0	0	0	0	0
	Totals	0	3	2	2	0	7
34	I	0	0	0	1	0	1
	F	0	0	0	0	0	0
	IB	0	0	0	0	0	0
	Totals	0	0	0	1	0	1
		Group B					
41	I	0	13	9	6	1	29
	F	0	2	1	2	4	9
	IB	0	3	3	3	0	9
	Totals	0	18	13	11	5	47
42	I	0	0	0	2	2	4
	F	0	0	0	0	1	1
	IB	0	3	3	0	0	6
	Totals	0	3	3	2	3	11
43	I	0	4	4	4	3	15
	F	0	0	0	2	1	3
	IB	0	3	3	0	3	9
	Totals	0	7	7	6	7	27
44	I	0	3	2	2	0	7
	F	0	0	2	2	2	6
	IB	0	0	3	3	3	9
	Totals	0	3	7	7	5	22
45	I	0	7	7	5	2	21
	F	0	0	2	2	3	7
	IB	0	3	3	3	0	9
	Totals	0	10	12	10	5	37
46	I	0	6	5	8	7	26
	F	0	0	0	2	3	5
	IB	0	3	3	3	3	12
	Totals	0	9	8	13	13	43

* I = Signs of inflammation; F = follicular hyperplasia; IB = inclusion bodies.

Table 6. *Experiment 2: rechallenge with MRC–4, 15 months after vaccination*

No. of baboons	Original vaccination	Mean score ($\sqrt{}$) at 28 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D. † ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
6	2 × 1·0 ml. s.c. 1 × 1·0 ml. i.v.	3·00	−1·29	1·14	1/6
5	3 × 1·0 ml. s.c.	3·88	−0·41	1·18	0/5
4	None	4·29	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper = 46, lower = 3.
* That is, with individual scores less than 3.
† L.S.D. = least signicant difference.

Table 7. *Experiment 2: comparison of complement-fixing antibody titres with conjunctival responses to challenge*

Baboon nos.	Route of vaccination	Reciprocal CF titres: days after 1st dose of vaccine			Cumulative score at 28 days after challenge
		24*	48	473	
29	2 s.c. + 1 i.v. dose	320	160	20	0
30		640	80	< 10	0
31		1280	640	10	4
32		250	320	20	8
33		1280	160	< 10	7
34		1280	640	< 10	1
	Means†	690	254	4	3·3
35	3 s.c. doses	1280	2560	20	2
36		1280	640	< 10	4
37		80	< 10	ND	7
38		450	640	< 10	21
39		120	640	< 10	11
40		640	320	< 10	26
	Means	407	245	4	11·8
41	No vaccine	< 5	40	ND	47
42		< 5	< 10	ND	11
43		< 5	20	< 10	27
44		< 5	160	30	22
45		< 5	20	< 10	37
46		< 5	ND	ND	43
	Means	< 5	19	3	31·2

* That is, on day of challenge.
† Means of antibody titres are geometric. ND = Not done.

Serological response to vaccination and challenge

Before vaccination, sera from all the animals used in this experiment failed at a dilution of 1/5 to fix complement with group antigen. Ten days after the final dose, i.e. on the day of first challenge, the antibody titres in the group receiving two subcutaneous and one intravenous dose ranged from 1/250 to 1/1280, and were on the average somewhat higher than in baboons given three subcutaneous doses (Table 7). By the sixth week after vaccination, serum titres in most of the vaccin-

ated animals had fallen 2- to 8-fold, but in some the titres had increased slightly. The finding at this time of small amounts of antibody in the control animals suggests that the increases were due to the stimulus of challenge; similar responses were observed in human volunteers inoculated with trachoma by the conjunctival route (Collier, Duke-Elder & Jones, 1958; 1960). By the time the second challenge was given, 15 months later, little or no complement fixing antibody was found in either vaccinated or control animals.

Table 7 also shows that there was no obvious inverse correlation between the formation of complement fixing antibody and the conjunctival response to challenge as measured by individual cumulative scores.

Antigen prepared in HeLa cells

Experiment 3: immunization with strain MRC–4 f: challenge with MRC–4

Although antigens grown in HeLa cells could not be used in man, the use of vaccines prepared from other cell cultures is a possibility; at present, only 'fast-killing' variants of TRIC agents can be readily propagated in cell cultures and, accordingly, this experiment was done to determine whether MRC–4 *f* grown in HeLa cells retained the immunogenicity of its parent strain. The minimum dose necessary to confer immunity was also investigated.

The vaccines were freshly prepared on the day of use as described under 'Materials and Methods'. On day 0, one group of five baboons received 0·5 ml. of undiluted vaccine subcutaneously into each axilla; this was repeated on day 7. On day 14, 1 ml. was given intravenously. The same schedule was followed for two more groups, which received respectively vaccine diluted 1/10 and 1/100 on each occasion. The undiluted vaccines contained respectively $10^{8\cdot0}$, $10^{7\cdot9}$ and $10^{8\cdot2}$ IFU/ml. and 167, 208 and 300 mg. total nitrogen per 100 ml.

Reactions to vaccination. A week after the first subcutaneous dose, all the baboons receiving undiluted vaccine had nodules approximately 2·5 cm. in diameter at the sites of injection; two of those receiving 1/10 vaccine had smaller nodules, but there were no local reactions in baboons inoculated with 1/100 vaccine. The nodules all regressed without necrosis during the following month.

The challenge inoculum prepared from the fourth yolk sac passage of MRC–4 had an infective titre of $10^{5\cdot9}$ EID 50/ml., and was inoculated into the right eyes of both vaccinated and control animals 11 days after the final intravenous dose of vaccine.

Response to conjunctival challenge

The total score for the group receiving undiluted vaccine was significantly less than that for the controls (Table 8), and two of out five animals showed convincing evidence of protection in terms of their individual scores. By contrast, the scores of animals receiving 1/10 vaccine did not differ significantly from those of the controls; nor was there evidence of protection in animals receiving 1/100 vaccine, one of whom had a score significantly higher than the upper 95% confidence limit for this experiment.

Serological response to vaccination

Before vaccination, sera from all the animals used in this experiment failed at a dilution of 1/5 to fix complement in the presence of group antigen. Table 9 shows that the mean antibody titres in animals receiving respectively 1/1 and 1/10 vaccine did not differ significantly; the mean serum titre of those given 1/100 vaccine was somewhat lower. As in the previous experiments, there was no relation between the titre of circulating antibody at the time of challenge and the severity of infection induced by conjunctival inoculation.

DISCUSSION

Methods for testing trachoma vaccines were recently reviewed by Collier (1966), who pointed out that none of the characteristics that can be assayed in the laboratory has yet been correlated with the results of field trials; and that since only primates appear to be susceptible to conjunctival inoculation with TRIC agents, experiments of the sort described here are, short of field trials in man, the most direct method available of ascertaining immunogenicity in relation to conjunctival infection. Nevertheless, the size and scope of such experiments are limited by expense and by the difficulty of handling large numbers of primates. To derive valid conclusions from comparatively small numbers of monkeys or baboons, in which there may be substantial individual variation in response, the clinical and microbiological examinations must be conducted in a uniform manner, and the results interpreted by a method that gives some assurance of objectivity.

Statistical analysis of the results of Expt. 2, previously reported in part by Collier (1961), confirmed that live MRC–4 grown in the yolk sac confers immunity to conjunctival challenge with a heavy dose of the homologous strain; and that in terms of the proportion of animals protected, 2 subcutaneous and 1 intravenous dose were more effective than 3 subcutaneous doses. However, a second challenge 15 months later showed that the immunity induced shortly after vaccination had largely disappeared even though the infective titre of the challenge inoculum was 10 times less than that on the first occasion. In this connexion, it is noteworthy that in trachomatous children the diminution in the severity of disease induced by vaccination is also short-lived (Collier *et al.* 1963).

Yolk-sac vaccines tested in simians by other workers usually consisted of inactivated TRIC agent, and will be discussed in the third paper of this series. There are, however, a few reports of the use of live vaccines in monkeys. For example, Grayston *et al.* (1960) contend that 4 successive doses of vaccine made from strain TW–29 modified the response to challenge with TW–10 given 2 weeks after the final dose; the route of immunization is not stated. Dawson *et al.* (1961) immunized small groups of rhesus monkeys with crude yolk-sac suspensions of various TRIC agents; they gave three intramuscular injections at weekly intervals, each containing approximately $10^{6.0}$ to $10^{7.7}$ ELD50/ml. In assessing the results, scores were assigned to the degree of follicular hyperplasia induced by challenge. By this criterion, and from examinations for inclusion bodies, they concluded that

Table 8. *Experiment 3: vaccination with aqueous suspensions containing graded doses of live MRC–4 f grown in HeLa cells: challenge with MRC–4*

No. of baboons	Dilution of vaccine	Mean score (√) at 28 days after challenge	Difference from mean score (√) of control group	L.S.D.† ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
5	1/1	3·28	−1·62	1·59	2/5
5	1/10	4·10	−0·80	1·59	0/5
6	1/100	5·27	+0·37	1·51	0/6††
6	No vaccine	4·90	—	—	—

95% confidence limits on scores for individual vaccinated animals: upper = 53, lower = 6.
* That is, with individual scores of 6 or less.
† L.S.D. = least significant difference.
†† One animal had a significantly high score (67).

Table 9. *Experiment 3: comparison of complement-fixing antibody titres with conjunctival responses to challenge*

Baboon nos.	Dilution of vaccine	Reciprocal CF titre 25 days after 1st dose of vaccine*	Cumulative score at 28 days after challenge
228	1/1	640	10
229		1280	24
230		640	15
231		1280	4
232		1280	6
		Means† 971	11·8
234	1/10	640	7
235		1280	35
236		640	14
237		1280	9
238		1280	27
		Means 971	18·4
240	1/100	1280	67
241		1280	19
242		640	21
243		1280	22
244		320	27
245		80	21
		Means 570	29·5
246	No vaccine	< 10	17
247		< 10	28
248		< 10	22
249		< 10	13
250		< 10	32
251		< 10	36
		Means < 10	24·7

* That is, on day of challenge
† Means of antibody titres are geometric.

strains BOUR, ASGH, Apache–1 and IC–Cal–1 modified the response to challenge with a small dose of BOUR given 8 days after the final dose of vaccine, but did not induce solid immunity.

Until now, there have been no reports of the use of TRIC vaccines prepared in cell cultures. Recent findings (Graham, 1965) suggest that the 'fast-killing' variant strains differ from their parent 'slow-killing' strains in the degree of cross-protection they give against pulmonary infection of mice; nevertheless, the 'fast-killing' strain MRC–4 *f* used for immunization in Expt. 3 induced immunity against its parent strain in two of five monkeys receiving undiluted vaccine. This vaccine was not titrated in chick embryos, but had an average titre of about 10^8 inclusion-forming units/ml. Taverne, Blyth & Reeve (1964*b*) showed that for *f* strains the infectivity titre in HeLa cells approximates to the 50% lethal dose in chick embryos (which for these strains is similar to the 50% infective dose) and that only about ten elementary bodies of *f* strains constitute one ELD50 for chick embryos; but with 'slow-killing' (*s*) strains, like that used for preparing the yolk sac vaccine, the ratio of total particles to infectivity is likely to be approximately 5000:1. Thus although the undiluted HeLa cell vaccine contained about 1000 times more infective TRIC agent than the yolk sac material and, by inference, a similar or greater number of elementary bodies, it was less effective in terms of immunogenicity against MRC–4. In an experiment to be described later, a HeLa cell vaccine with an infective titre approximately 10 times higher than that of the vaccines used in the tests reported here protected all of six baboons against conjunctival challenge with the parent *s* strain.

The serological results suggest that the serum titre of PLT group complement fixing (CF) antibody is not directly related, if it is related at all, to the conjunctival response to infection. Similar findings have been reported by others; thus Bietti, Guerra, Felici & Vozza (1962) and Khaw *et al.* (1963) found no correspondence between the titres of CF antibodies in vaccinated human volunteers and the course of conjunctival infection after challenge with TRIC agent. Collier *et al.* (1963) observed that the beneficial effect of vaccination on a proportion of children with active trachoma was unrelated to CF antibody titre. It is noteworthy that Blyth, Reeve, Graham & Taverne (1962) concluded from studies on rabbits immunized with TRIC agent that 'production of antibody fixing complement with group antigen does not parallel that of neutralizing antibody'. Nevertheless, they found that sera with low titres of CF antibody were unlikely to contain much neutralizing antibody. The animals in our experiments were not tested for neutralizing antibody; but in these and other experiments to be reported subsequently, it is interesting that a number of baboons that were protected against conjunctival challenge had comparatively low titres of CF antibody.

Our observation that CF antibodies induced by vaccination do not persist for long confirms the findings of Grayston *et al.* (1963) and Collier *et al.* (1963) in children given experimental trachoma vaccines.

SUMMARY

Parallel titrations of a strain of trachoma (MRC–221) and one of inclusion conjunctivitis (MRC–4) in the baboon conjunctiva and in chick embryos suggest that ten to twenty 50% egg infective doses are equivalent to one 50% baboon infective dose; but that at least 1000 egg infective doses are needed to induce moderate or severe infections in all of a given number of baboons.

For vaccine experiments in baboons, a system of scoring physical signs and presence of inclusion bodies was devised; the significance of differences in vaccinated and control animals in their response to conjunctival challenge was determined by analysis of variance. An aqueous suspension of live MRC–4 grown in the yolk sac was given as two subcutaneous doses and one intravenous dose at weekly intervals, and protected all of six baboons challenged with the homologous strain; three similarly spaced subcutaneous doses were less effective. The immunity induced by this vaccine waned considerably during the ensuing 15 months. Vaccine prepared from a live 'fast-killing' variant of MRC–4 grown in HeLa cells was less effective than MRC–4 itself in protecting baboons against infection with the parent strain.

Although both yolk sac and HeLa cell vaccines induced the formation of antibody fixing complement with trachoma group antigen, the serum titres in individual animals at the time of challenge were unrelated to the degree of protection; during a 15 month observation period there were pronounced falls in the titres of antibody induced either by vaccination or by challenge with egg-grown TRIC agent.

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Immunogenicity of experimental trachoma vaccines in baboons.

II. Experiments with adjuvants, and tests of cross-protection

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INTRODUCTION

In the preceding paper (Collier & Blyth, 1966) we showed that although aqueous suspensions of strain MRC–4 grown in the chick embryo yolk sac protected baboons against conjunctival challenge shortly after vaccination, little or no immunity was demonstrable a year or so later; the investigation of adjuvants reported here was undertaken in an attempt to improve the immunogenicity of the vaccine.

As well as duration of immunity, an important consideration in devising a trachoma vaccine is the extent of cross-protection between different strains. In baboons, several trachoma agents proved insufficiently pathogenic for use as conjunctival challenge (Collier, 1961, 1962); but a few strains, notably MRC–187. were later found to induce moderately severe infections, and a full cross-protection test was made with this strain and the MRC–4 strain of inclusion conjunctivitis. MRC–187 was also used to test the immunity induced by an oil adjuvant trachoma vaccine against challenge with the homologous strain.

The trachoma/inclusion conjunctivitis (TRIC) agents used in these experiments are shown in Table 1. Except where otherwise stated, the experimental and statistical methods are those described previously (Collier & Blyth, 1966).

Table 1. *Designation of trachoma agents used in Experiments* 4–9

Full designation	Abbreviation	Reference
TRIC/ /WAG/MRC–17/OT (formerly G 17)	MRC–17	Sowa & Collier, 1960
TRIC/ /WAG/MRC–1/OT (formerly G 1)	MRC–1/OT	Collier & Sowa, 1958
TRIC/ /WAG/MRC–187/OT	MRC–187	—
TRIC/ /WAG/MRC–062/OT	MRC–062	—

EXPERIMENTS WITH MINERAL OIL ADJUVANTS

Experiments 4–7 were parallel tests of the immunogenicity of 'aqueous' vaccines and mineral oil adjuvant vaccines prepared from them.

Aqueous vaccines were partially purified suspensions of live TRIC agent in phosphate buffered saline (PBS) (Dulbecco & Vogt, 1954) or in sucrose potassium glutamate (SPG) (Bovarnick, Miller & Snyder, 1950). They were stored in 1·0 ml.

amounts at −60° C. until required. Their characteristics are summarized in Tables 2 and 4.

Adjuvant vaccines. Nine volumes of light mineral oil (Drakeol 6 VR, Pennsylvania Refinery Company) were mixed with 1 volume of an emulsifying agent, mannide monooleate (Arlacel A, Atlas Powder Company); the mixture was sterilized by filtration at 7 lb./sq.in. positive pressure through a Gradocol membrane (A.P.D. 0·6 μ). On the day of use, 1 vol. aqueous vaccine, thawed rapidly from −60° C., was added to 1 vol. Drakeol/Arlacel A mixture; except in Expt. 7 (see below) the vaccine was emulsified in an M.S.E. blender at 2000 rev./min. for 3 min.

Table 2. *Aqueous yolk-sac vaccines used for Experiments* 4–6

	Experiment no.		
	4	5	6
Strain	MRC–187	MRC–187	MRC–062
Chick embryo passage	11	6	4
Infectivity titre ($\log_{10}$ EID50/ml.)	4·4	4·8	5·2
Total particle count ($\log_{10}$/ml.)	9·4	9·8	11·9
Reciprocal titre of complement-fixing group antigen	ND	1280	512
Total nitrogen (mg./100 ml.)	31·5	216·0	ND

EID50 = 50 % egg infective dose.
ND = not done.

at 4° C. To ensure that a true water-in-oil emulsion had formed, vaccines were subjected to the 'drop test', whereby a drop of this type of emulsion retains its integrity when expelled on to the surface of cold water. They were also tested for stability on horizontal centrifugation at 380 ***g*** for 10 min.

Challenge inocula were aqueous suspensions of 'slow-killing' TRIC agents (Reeve & Taverne, 1963) grown in the yolk sac.

Experiment 4*: vaccination with live MRC–*187 *grown in yolk sac; challenge with MRC–*187

Aqueous vaccine. On days 0 and 7, five baboons were given 0·5 ml. subcutaneously into the thoracic surface of both axillae; an intravenous dose of 1·0 ml. was given on day 14.

Adjuvant vaccine. On day 0, five baboons were each given 0·25 ml. intramuscularly into each buttock. The total dose of 0·50 ml. was equivalent to 0·25 ml. of aqueous vaccine.

Control vaccines. Three animals received dummy aqueous vaccine, and three more were given dummy adjuvant vaccine, prepared from normal yolk sac and given in the dosages used for the vaccines proper.

Challenge. On day 24, all baboons were challenged by conjunctival inoculation of MRC–187 in its 11th yolk sac passage, with an infectivity titre of $10^{6·7}$ EID50/ml.

Results

The scores for both control groups were similar, and are considered together in Table 3, which shows that neither aqueous nor adjuvant vaccine exerted a protective effect; indeed, two animals receiving adjuvant vaccine had high scores suggesting an enhanced response to challenge.

Table 3. *Experiment* 4*: vaccination with aqueous and mineral oil suspensions of live MRC*–187 *grown in yolk sac: challenge with MRC*–187

Nos. of baboons	Vaccine	Mean score ($\sqrt{}$) at 29 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D.‡ ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
5	Aqueous	3·71	−0·60	0·98	0/5
5	Adjuvant	5·89	+1·58	0·98	0/5†
6	Normal yolk sac	4·31	—	—	—

95% confidence limits on scores for individual vaccinated animals: upper, 45; lower, 3.
* That is, with individual scores of 3 or less.
† Two animals had significantly high scores (45 and 48).
‡ L.S.D. = least significant difference.

Experiment 5*: vaccination with live MRC*–187 *grown in yolk sac: challenge with MRC*–187

The vaccines and challenge inoculum were similar to those used in Expt. 4, except that the antigen was made from the 6th chick embryo passage (Table 2).

Aqueous vaccine was given to six baboons as $2 \times 0{\cdot}5$ ml. doses subcutaneously on days 0, 8 and 14, followed by 0·5 ml. intravenously on day 38.

Adjuvant vaccines were given to six baboons in a single intramuscular dose of 0·5 ml. (equivalent to 0·25 ml. aqueous vaccine) on day 0.

Controls. As in the previous experiment, two groups of three baboons received respectively dummy aqueous and adjuvant vaccines prepared from normal yolk sac.

Challenge. In Expt. 4, the failure of the adjuvant vaccine to protect might have been due to premature challenge before immunity had developed; in Expt. 5, challenge was delayed until day 70. The suspension of MRC–187 used was 11th yolk sac passage material with a titre of $10^{6{\cdot}3}$ EID50/ml.

Results

There was again no evidence of protection either by aqueous or by adjuvant vaccine; on this occasion no vaccinated animals had scores significantly higher than those of the controls.

Experiment 6*: vaccination with live MRC*–062 *grown in yolk sac; challenge with MRC*–4

Aqueous vaccine was given intramuscularly to five baboons on days 0, 14 and 28; each dose of 1·0 ml. was given as 0·5 ml. into each buttock.

Adjuvant vaccine was given to two groups each of five baboons. One group was given $2 \times 0{\cdot}5$ ml. intramuscularly on day 0, as for aqueous vaccine; the other received a second similar dose on day 28. This vaccine was serially diluted in SPG

and titrated in chick embryos; allowing for its two-fold dilution, the titre of $10^{4.7}$ EID50/ml. was close to that of the aqueous vaccine (Table 2), and showed that there was no inactivation of TRIC agent during the emulsifying process.

Control animals received no vaccine.

Challenge. All animals were inoculated on day 71 with pooled 2nd and 5th passage MRC–4. The titration in chick embryos of this material was unsatisfactory, but it induced infection of adequate severity in the baboon conjunctiva.

Results

For all the vaccinated animals, the group scores were higher than for the controls, but not significantly so. One animal receiving two doses of adjuvant vaccine had an individual score significantly higher than the upper 95% confidence limit for this experiment.

Rechallenge after 11 months

The baboons that had received two doses of adjuvant vaccine and the control group were again challenged 11 months after the first dose of vaccine to determine whether late immunity had developed. The inoculum was MRC–4 in its 4th yolk sac passage with an infectivity titre of $10^{5.9}$ EID50/ml. There were again no significant differences between the scores of the vaccinated and control animals.

Experiment 7: vaccination with live MRC–4 f grown in HeLa cells; challenge with MRC–4

The object of this experiment was to determine whether the rather disappointing result with aqueous HeLa cell vaccine (Collier & Blyth, 1966) could be improved by using a higher concentration of MRC–4 *f* or by incorporating a mineral oil adjuvant. It was done before Expts. 4–6, and made use of a different method for preparing adjuvant vaccine.

Aqueous vaccines were prepared from heavily infected HeLa cell monolayers (Collier & Blyth, 1966). On days 0 and 8, six baboons each received $2 \times 0{\cdot}5$ ml. subcutaneously, given as in Expt. 4, and 1·0 ml. intravenously on day 15. The characteristics of the vaccines, which were made freshly on each occasion, are given in Table 4.

Adjuvant vaccines were prepared by repeatedly expelling the mixed oil and aqueous phases from a 10 ml. syringe fitted with a no. 1 needle. The first use of this method resulted in an unstable oil-in-water (O/W) emulsion, which was nevertheless given intramuscularly to a group of six baboons; on day 0 each received 0·5 ml. of adjuvant vaccine prepared from batch 1 into each buttock.

On day 8 a fresh lot of adjuvant vaccine was prepared by the syringe method from batch 2 of the aqueous suspension; this time a stable water-in-oil (W/O) emulsion resulted, and was inoculated intramuscularly ($2 \times 0{\cdot}5$ ml.) into six more baboons.

Controls. On day 8 six baboons were each given $2 \times 0{\cdot}5$ ml. of a dummy W/O vaccine made from uninfected HeLa cells, containing 145 mg. total nitrogen per 100 ml.

Challenge. On day 40 all animals were challenged in their right eyes with MRC–4 in its 3rd yolk-sac passage; the infectivity titre was $10^{4\cdot1}$ EID50/ml.

Table 4. *Aqueous vaccines used for Experiment 7: prepared from live MRC–4 f grown in HeLa cells*

	Batch no.		
	1	2	3
Infectivity titre			
(*a*) $\log_{10}$EID50/ml.	9·0	9·3	8·6
(*b*) $\log_{10}$IFU/ml.	8·7	9·7	9·1
Total particle count ($\log_{10}$/ml.)	10·1	10·3	10·2
Reciprocal titre of complement-fixing group antigen	2560	2560	ND
Total nitrogen (mg./100 ml.)	267	834	334

EID50 = 50% egg infective dose.
IFU = inclusion-forming units.
ND = not done.

Table 5. *Experiment 7: vaccination with aqueous and mineral oil suspensions of live MRC–4 f grown in HeLa cells; challenge with MRC–4*

No. of baboons	Vaccine	Mean score ($\sqrt{}$) at 28 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D.† ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
6	Aqueous	2·26	−3·61	1·60	6/6
6	Adjuvant (oil-in-water)	4·00	−1·87	1·60	2/6
6	Adjuvant (water-in-oil)	4·21	−1·66	1·60	3/6
6	No vaccine	5·87	—	—	—

95% confidence limits on scores for individual vaccinated animals: upper, 68; lower, 12.
* That is, with individual scores of 12 or less.
† L.S.D. = least significant difference.

Results

All the baboons given aqueous vaccine were protected to a significant extent against conjunctival challenge with MRC–4, but the adjuvant vaccines were less effective (Table 5). The oil-in-water suspension protected only two of six animals, and the correctly prepared water-in-oil emulsion was little better, since it failed to protect half the baboons receiving it.

Rechallenge after 11 months

Eleven months after the 1st challenge, the left eyes of all surviving animals were inoculated with the 3rd yolk-sac passage of MRC–4; the infectivity titre was $10^{4\cdot2}$ EID50/ml. None of the vaccinated animals resisted the second challenge and immunity was not prolonged by using a mineral oil adjuvant (Table 6).

Table 6. *Experiment 7: rechallenge with MRC–4, 11 months after vaccination*

No. of baboons	Vaccine	Mean score ($\sqrt{}$) at 28 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D.† ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
5	Aqueous	4·84	+0·25	1·68	0/5
4	Adjuvant (oil-in-water)	4·50	−0·09	1·79	0/4
6	Adjuvant (water-in-oil)	5·42	+0·83	1·60	0/6
6	No vaccine	4·59	—	—	—

95% confidence limits on scores for individual vaccinated animals: upper, 49; lower, 4.
* That is, with individual scores of 4 or less.
† L.S.D. = least significant difference.

Table 7. *Experiments with mineral oil adjuvants: titres of complement-fixing antibody at times of challenge*

Expt. no.	Vaccine	Strain	Mean CF titre* at time of: 1st challenge	2nd challenge
4	Aqueous	MRC–187	< 5	—
	Adjuvant	MRC–187	15	—
	Aqueous	Normal yolk sac	< 5	—
	Adjuvant	Normal yolk sac	< 5	—
5	Aqueous	MRC–187	39	—
	Adjuvant	MRC–187	36	—
	Aqueous	Normal yolk sac	0	—
	Adjuvant	Normal yolk sac	3	—
6	Aqueous	MRC–062	2	ND
	Adjuvant (1 dose)	MRC–062	26	ND
	Adjuvant (2 doses)	MRC–062	92	67
	None	MRC–062	< 5	2
7	Aqueous	MRC–4 *f*	2370	113
	Adjuvant (oil-in-water)	MRC–4 *f*	4560	640
	Adjuvant (water-in-oil)	MRC–4 *f*	3225	508
	None	MRC–4 *f*	7	58

* Reciprocal of geometric mean titre of antibody fixing complement with group antigen.
ND = not done.

Serological findings in Experiments 4–7

Tests for psittacosis–lymphogranuloma–trachoma (PLT) group complement-fixing (CF) antibody were made as described by Collier & Blyth (1966). The sera of animals immunized with yolk sac vaccines were tested with antigen prepared in HeLa cells, and yolk sac antigen was used for testing sera from animals immunized with HeLa cell vaccine. Table 7 gives the geometric mean titres of sera from vaccinated and control groups at the time of challenge. None of the aqueous yolk

sac vaccines prepared from strains MRC–187 or MRC–062 induced high titres of CF antibody, and little advantage was gained by using mineral oil adjuvant, although a second dose of adjuvant vaccine (Expt. 6) resulted in a somewhat higher mean titre.

The HeLa cell vaccines prepared from MRC–4*f* (Expt. 7) induced circulating CF antibody in high titres. The mineral oil suspensions performed somewhat better than the aqueous vaccine, but the difference was not pronounced; it is interesting that the physical state of the emulsion had little influence in this respect.

Sera from the baboons receiving two doses of adjuvant vaccine (Expt. 6) were again tested 11 months after vaccination, and still contained small amounts of antibody. Over a similar period there were pronounced falls in titre in the baboons given HeLa cell vaccine (Expt. 7); and whereas the mean titre in the group given aqueous vaccine fell by a factor of 20, there was a 65- to 70-fold fall in both the groups vaccinated with mineral oil suspensions.

In none of these experiments was there any relation between the CF titres in individual baboons at the time of challenge and their conjunctival responses.

TESTS OF CROSS-PROTECTION, AND USE OF PRECIPITATED ANTIGENS

Experiments 8 and 9 were made primarily to determine whether two more trachoma agents, MRC–17 and MRC–1/OT, would protect against challenge with the inclusion conjunctivitis strain MRC–4. The opportunity was also taken to test the immunogenicity of TRIC agents precipitated either by protamine (Chambers & Henle, 1941) or by calcium phosphate.

Experiment 8: vaccination with live MRC–17 grown in yolk sac: challenge with MRC–4

Aqueous vaccine. Twenty-four grams (wet weight) of heavily infected yolk sacs were shaken in 320 ml. of PBS for 30 min. at 4° C. The suspension was strained through several layers of surgical gauze to remove gross solid material and divided into two equal parts, one of which was partially purified and concentrated by differential centrifugation to give a final volume of 30 ml. aqueous vaccine in SPG.

Protamine-precipitated (Pr-P) vaccine. The remaining 160 ml. of crude suspension was mixed with an equal volume of Seitz-filtered 2% (w/v) protamine sulphate, and stood at 4° C. for 10 min. The resulting precipitate was deposited by horizontal centrifugation at 170 *g.* for 10 min., and resuspended in 30 ml. SPG

Both vaccines were sealed in 1·2 ml. amounts in ampoules and stored at −70° C. until required. The infectivity titre of the aqueous vaccine ($10^{3·6}$ EID50/ml.) was lower than usual in these experiments, and a further loss in titre took place as a result of protamine treatment (Table 8).

Vaccination. Two groups each of six baboons were vaccinated respectively with aqueous and Pr–P vaccine; subcutaneous and intravenous injections were given according to the schedule for aqueous vaccine in Expt. 4. Six unvaccinated baboons served as controls.

Challenge. The inoculum was a pool of 2nd and 4th passage MRC–4 grown in the yolk sac, with an infectivity titre of $10^{4.9}$ EID50/ml. It was administered on day 24, i.e. 10 days after the final dose of vaccine.

Table 8. *Vaccines used for Experiments* 8 *and* 9

Experiment no.	8		9	
Strain	MRC–17		MRC–1/OT	
Chick embryo passage	48		32	
Vaccine	Aqueous	Protamine	Aqueous	Phosphate
Infectivity titre ($\log_{10}$ EID50/ml)*	3·6	3·0	5·8	4·9

* EID50 = 50 % egg infective dose.

Table 9. *Experiment* 8*: vaccination with suspensions of live MRC–*17 *grown in yolk sac: challenge with MRC–*4

No. of baboons	Vaccine	Mean score (√) at 28 days after challenge	Difference from mean score (√) of control group	L.S.D.† (P = 0·05)	No. protected* / No. vaccinated
6	Aqueous suspension	3·98	− 1·83	1·28	2/6
6	Protamine-precipitated antigen	4·44	− 1·37	1·28	2/6
6	No vaccine	5·81	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper, 67; lower, 11.
* That is, with individual scores of 11 or less.
† L.S.D. = least significant difference.

Results

There was no difference between the aqueous and Pr–P vaccines in terms of immunogenicity (Table 9). Both were partially effective in that they each protected 2/6 baboons against challenge with MRC–4.

Experiment 9*: vaccination with live MRC–*1*/OT grown in yolk sac: challenge with MRC–*4

Aqueous vaccine. Forty-nine grams (wet weight) of infected yolk sacs were shaken with 120 ml. of PBS and strained through gauze as described for Expt. 8. The crude suspension was centrifuged at 140 ***g***. for 10 min. to remove coarse particles; the elementary bodies were then deposited by angle centrifugation at 11,000 ***g*** for 30 min, at 4° C. Half the deposit resuspended in 50 ml. SPG constituted the aqueous vaccine, which was stored at − 70° C. in 1·2 ml. amounts.

Phosphate-precipitated (Ph-P) vaccine. The use of calcium-phosphate columns for purifying viruses was suggested by Taverne, Marshall & Fulton (1958). In our experiments, the brushite form of the salt ($CaHPO_4.2H_2O$) was prepared by the method of Tiselius, Hjertén & Levin (1956) and washed in 0·2 M phosphate buffer, pH 7·0. The remaining half of the angle deposit was suspended in 15 ml. of the

same buffer, to which were added 15 ml. freshly precipitated calcium phosphate. After standing at room temperature for 15 min., with stirring at 5 min. intervals, the mixture was centrifuged at 170 *g* for 5 min.; the deposit was retained, and the supernatant was treated twice more with calcium phosphate; the total amount used was 44 g., equivalent to 4 g. per yolk sac. The final supernatant was discarded; the pooled deposits (44 ml.) were made up to 60 ml. with SPG, and stored in 1·2 ml. amounts at −70° C. During purification, progressive removal of elementary bodies from successive supernatant fluids was verified by examining Castañeda-stained smears.

The infectivity titre of the Ph-P vaccine was about 10 times less than that of the aqueous material (Table 8).

Vaccination. The aqueous vaccine was given to six baboons according to the schedule used for Expt. 4, except that the final intravenous injection was given on day 21. The Ph-P vaccine was also given to six baboons on days 0, 7 and 21, but as three subcutaneous doses each of 2·0 ml. (equivalent to 1·0 ml. of aqueous vaccine). The six control animals received no vaccine.

Challenge. The inoculum was a pooled suspension of the 2nd and 3rd yolk sac passages of MRC-4, with an infectivity titre of $10^{5\cdot0}$ EID50/ml.; it was inoculated on day 31, 10 days after the final dose of vaccine.

Results

Neither vaccine protected against challenge with MRC–4. One animal receiving the aqueous suspension appeared to have an enhanced response to challenge (Table 10).

Table 10. *Experiment* 9*: vaccination with suspensions of live MRC–*1*/OT grown in yolk sac; challenge with MRC–*4

No. of baboons	Vaccine	Mean score (√) at 20 days after challenge	Difference from mean score (√) of control group	L.S.D.‡ (P = 0·05)	No. protected* / No. vaccinated
6	Aqueous suspension	4·52	+0·55	1·27	0/6†
6	Phosphate-precipitated antigen	5·04	+1·07	1·27	0/6
6	No vaccine	3·97	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper, 41; lower, 2.
* That is, with individual scores of 2 or less.
† One animal had a significantly high score (43).
‡ L.S.D. = least significant difference.

Experiment 10*: cross-protection test with live MRC–*4 f *and MRC–*187 *grown in the yolk sac*

The live aqueous vaccines were partially purified by centrifugation, and further purified by treatment with molar KCl as described by Collier & Blyth (1966) for the preparation of group antigen. The infectivity titre of the MRC–4 *f* suspension

was originally $10^{6.9}$ EID50/ml.; it was diluted 20-fold to make it comparable in this respect with the MRC–187 vaccine (Table 11). After dilution, its total particle count was 100 times less than the MRC–187 suspension.

Vaccination. Both vaccines were given subcutaneously to groups of baboons on days 0 and 7, and intravenously on day 14 by the method described in Expt. 4. Groups A and B received MRC–4 *f*, and groups C and D were given MRC–187 vaccine. Two control groups (E and F) were left unvaccinated.

Challenge. The MRC–4 inoculum was prepared from the 5th yolk sac passage, and contained $10^{4.3}$ EID50/ml. The MRC–187 challenge consisted of the vaccine itself diluted to this infectivity titre. On day 24 groups A, C and E were challenged with MRC–4, and groups B, D and F with MRC–187; all inoculations were made into the right eyes.

Table 11. *Vaccines used for Experiment* 10

	Strain	
	MRC–4 *f*	MRC–187
Chick embryo passage	19	12
Infectivity titre ($\log_{10}$ EID50/ml.)	5·6	5·7
Total particle count ($\log_{10}$/ml.)	8·1	10·2

EID50 = 50% egg infective dose.

Table 12. *Experiment* 10*: cross-protection test with live MRC–*4 f *and MRC–*187 *grown in yolk sac*

Group	No. of baboons	Vaccine strain	Mean score (√) at 28 days after challenge	Difference from mean score (√) of control group	L.S.D.† (P = 0·05)	No. protected* / No. vaccinated
		(*a*) Challenge with MRC–4				
A	5	MRC–4 *f*	3·48	−2·21	1·65	3/5
C	5	MRC–187	4·63	−1·06	1·65	0/5
E	5	No vaccine	5·69	—	—	—
		(*b*) Challenge with MRC–187				
B	5	MRC–4 *f*	3·99	−1·64	1·75	1/5
D	5	MRC–187	3·71	−1·92	1·75	2/5
F	4	No vaccine	5·63	—	—	—

95% confidence limits on scores for individual vaccinated animals (*a* and *b*): upper, 66; lower, 10.

* That is, with individual scores of 10 or less.

† L.S.D. = least significant difference.

Results

In terms of the mean group scores, the severity of infection induced in the controls by both challenges was very similar (Table 12). MRC–4 *f* protected 3 of 5 baboons challenged with MRC–4, but only 1 of 5 challenged with MRC–187; and whereas MRC–187 vaccine immunized 2 of 5 baboons against challenge with the homologous strain, it failed completely against challenge with MRC–4.

Rechallenge after 10 months

All surviving animals were challenged in their left eyes with the appropriate TRIC agents; the MRC–4 inoculum was made from the 6th yolk-sac passage, and had an infectivity titre of $10^{5\cdot0}$ EID50/ml.; the MRC–187 challenge was prepared from the 13th yolk-sac passage, and contained $10^{4\cdot3}$ EID50/ml.

Results

As in previous experiments, any immunity originally induced by either vaccine was no longer demonstrable (Table 13).

Table 13. *Experiment 10: rechallenge 10 months after vaccination*

Group	No. of baboons	Vaccine strain	Mean score ($\sqrt{}$) at 28 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D.† ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
			(*a*) Challenge with MRC–4			
A	5	MRC–4*f*	5·61	+0·77	1·17	0/5
C	5	MRC–187	5·75	+0·91	1·17	0/5
E	3	No vaccine	4·84	—	—	—
			(*b*) Challenge with MRC–187			
B	5	MRC–4*f*	5·27	−0·14	1·17	0/5
D	5	MRC–187	5·88	+0·47	1·17	0/5
F	4	No vaccine	5·41	—	—	—

95% confidence limits on scores for individual vaccinated animals. (*a*) Upper, 58; lower, 4. (*b*) Upper, 67; lower 7.

* That is, with individual scores of (*a*) 4 or less; (*b*) 7 or less.

† L.S.D. = least significant difference.

Serological results in Experiments 8–10

Table 14 gives the mean serum titres of CF antibody in vaccinated and control groups, tested on the day of challenge. None of the aqueous yolk-sac vaccines prepared from strains MRC–17, MRC–1/OT, MRC–4*f* or MRC–187 induced high titres of CF antibody. In Expt. 10 there was a pronounced difference between groups A and B in terms of mean serum titre; the reason is not clear, since both groups received the same vaccine in identical dosage. Eleven months later, however, the titres in both groups had fallen to the same low values.

The amounts of CF antibody elicited by protamine-precipitated and by untreated aqueous MRC–17 vaccines were similar; but the phosphate-precipitated MRC–1/OT vaccine induced higher CF titres than did its aqueous counterpart. Again, there was no relation between individual CF antibody response and the course of conjunctival infection after challenge.

DISCUSSION

Whereas successive subcutaneous and intravenous doses of an aqueous suspension of MRC–4*f* protected all of six baboons against challenge with MRC–4, 1·0 ml. of adjuvant vaccine given intramuscularly was less effective, and a water-

in-oil emulsion performed little better than an oil-in-water preparation (Expt. 7). By contrast with MRC–4 (Collier & Blyth, 1966), strain MRC–187 proved to be a comparatively poor immunogen, even when given as an aqueous suspension and tested against challenge with the homologous strain; and combination with oil adjuvant failed to improve it. Oil adjuvant also failed to render MRC–062 immunogenic against challenge with MRC–4.

Table 14. *Tests of cross-protection, and of precipitated antigens; titres of complement-fixing antibody at times of challenge*

Expt. no.	Vaccine	Strain	Mean CF titre* at time of: 1st challenge	2nd challenge
8	Aqueous	MRC–17	28	—
	Protamine-precipitated		34	—
	None		1	—
9	Aqueous	MRC–1/OT	10	—
	Phosphate-precipitated		160	—
	None		2	—
10	Group A	MRC–4 *f*	14	7
	Group B		147	8
	Group C	MRC–187	2	4
	Group D		3	5
	Group E	None	< 5	16
	Group F		< 5	2

* Reciprocal of geometric mean titre of antibody fixing complement with group antigen.

In the absence of any adjuvant effect, the comparatively poor performance of the mineral oil vaccines was probably due to their being given in lower dosage than the aqueous suspensions, and by a different route. Emulsification with oil and Arlacel did not seem to affect the antigen itself, at least in terms of viability (Expt. 6). There was no evidence that the poor results with oil adjuvant were due to premature challenge; and, as judged by a second challenge 11 months later, there was no indication that baboons successfully immunized with adjuvant vaccine retained their immunity longer than those protected by aqueous suspensions.

As well as being a poor immunogen, MRC–187 induced little or no circulating CF antibody; MRC–062 also failed in this respect. Since the vaccines were comparable in infectivity titre with a yolk sac vaccine made from MRC–4 which induced high CF titres (Collier & Blyth, 1966), it seems that MRC–062 and MRC–187 are inherently incompetent at inducing formation of PLT group CF antibody. The addition of mineral oil adjuvant was of little value in this respect, and did not prolong the persistence of circulating antibody. The MRC–4 *f* vaccine prepared in HeLa cells induced higher titres than the MRC–4 yolk sac vaccine previously described; this was probably due to its greater content of antigen.

The lack of relationship between CF antibody response and the course of infection following challenge confirms our previous findings (Collier & Blyth, 1966).

Our observation that mineral oil adjuvant failed to increase the immunogenicity of TRIC antigens agrees with that of Snyder *et al.* (1964), who found that whereas an aqueous vaccine inactivated with formalin significantly diminished the trachoma attack rate in Saudi Arabian children, the same batch of suspension emulsified with Drakeol 6 and Arlacel A did not. By contrast, Grayston, Wang, Woolridge & Alexander (1964) found that mineral oil adjuvant improved the immunogenicity of a trachoma vaccine tested in monkeys against challenge with the homologous strain. They maintain that mineral oil behaves as an adjuvant only if the vaccine contains at least 10^8–10^9 elementary bodies, a condition that was met in our own experiments. Khaw *et al.* (1963) contend that mineral oil adjuvant prolonged the CF antibody response to trachoma vaccine in human volunteers, but they made no direct comparison with aqueous vaccine prepared from the same suspension. They also stated that adjuvant vaccine modified the response of volunteers to conjunctival challenge, without however reducing the infection rate. Like us, they found no relationship between CF antibody titre and severity of response to conjunctival challenge. From the results of field trials in Ethiopia, Bietti (1964) claims that a single dose of oil adjuvant vaccine had no effect, but that an additional dose of aqueous vaccine 45 days later diminished the trachoma attack rate in healthy subjects, and favourably modified the course of established trachoma. There is therefore no general agreement about the value of oil adjuvants for trachoma vaccines. The conflicting reports suggest that much more remains to be learnt about the methods of making and administering preparations of this nature; and in particular, the mass of antigen required, its degree of purity and the influence of particle size remain to be determined.

In the cross-protection tests, MRC–17 afforded better protection against conjunctival challenge with MRC–4 than did an MRC–1/OT vaccine with a higher infectivity titre; the challenge dose of MRC–4 used in both experiments (nos. 8 and 9) was similar. In these experiments, both protamine sulphate and calcium phosphate were used primarily in attempts to purify and concentrate the antigen; it was hoped that calcium phosphate might also act as an adjuvant, but it proved disappointing in this respect.

In the cross-protection experiment with MRC–4*f* and MRC–187 (Expt. 10) the vaccines were made comparable in terms of infectivity titre, but it should be noted that the MRC–187 vaccine contained 100 times more elementary bodies. The challenge inocula prepared respectively from MRC–4 and MRC–187 had the same infectivity titres and induced infections of comparable severity in the control animals. MRC–4*f* protected 3 of 5 animals against challenge with MRC–4, but only 1 of 5 against MRC–187; and whereas MRC–187 vaccine conferred partial protection against challenge with the homologous strain, it failed completely to protect against MRC–4. These findings may be supplemented with data from the other experiments. For example, the failure of MRC–4*f* to protect all the animals challenged with MRC–4 was probably due to insufficient dosage (cf. Expt. 7). This also seems to explain the failure of aqueous MRC–187 vaccines to protect against homologous challenge in Expts. 4 and 5, since a vaccine with a rather higher infectivity titre and total particle count protected 2 of 5 baboons in Expt. 10.

It is not yet clear whether the infectivity titre or the total particle count is the more important factor in determining the critical dose of live vaccine necessary to confer protection. Apart from considerations of dosage, however, there are clear indications of antigenic differences between the TRIC agents used in these tests. Thus the Gambian trachoma strains MRC–1/OT and MRC–17 differed in their ability to protect against a strain of inclusion blennorrhoea (MRC–4); for a given dose of live vaccine, MRC–1/OT was less effective than the homologous strain in protecting against MRC–4 (cf. expt. 2, Collier and Blyth, 1966); and the findings in Expt. 10 suggest that there is an antigenic difference between MRC–4*f* and MRC–187.

Antigenic differences between PLT agents can be demonstrated by the mouse toxin protection test (Bell & Theobald, 1962; Wang & Grayston, 1963) and by immunofluorescence (Hanna & Bernkopf, 1964; Nichols & McComb, 1964). None of the combinations of strains used in our experiments have been tested by these methods; but Grayston *et al.* (1964) obtained evidence suggesting that the serological grouping elicited by the mouse toxin test is reflected by the results of cross-protection tests in the monkey conjunctiva.

The results of the second challenges in Expts. 7 and 10 confirmed our previous observation that the immunity induced in baboons by aqueous vaccines is of comparatively short duration.

The serological findings in Expts. 8 and 10 confirmed that MRC–187 does not readily call forth group complement-fixing antibodies, and showed that two more Gambian strains, MRC–17 and MRC–1/OT, resemble it in this respect; but the addition of calcium phosphate to the MRC–1/OT vaccine resulted in rather higher antibody titres. In Expt. 10, the antibody response to aqueous MRC–4*f* vaccine was much less than in Expt. 7, probably because of the smaller dose of antigen. Again, there was no relation between individual antibody titres at the time of challenge and response to conjunctival inoculation.

In Expts. 4, 6 and 9, three baboons receiving mineral oil adjuvant vaccines and one given aqueous vaccine had significantly enhanced responses to conjunctival challenge administered comparatively soon after vaccination. This reaction seems to be induced only by relatively ineffective vaccines; it has not been observed in baboons challenged a second time a year or so after vaccination. It appears that vaccines that fail to confer solid protection may induce instead a short-lived state of hypersensitivity; and that, like immunity, this state is unrelated to the titre of circulating CF antibody. Similar findings are reported by Grayston, Woolridge & Wang (1962) who found that in monkeys large single doses of a trachoma vaccine combined with aluminium hydroxide adjuvant resulted in an enhanced response to conjunctival challenge, whereas repeated small doses without adjuvant afforded partial protection. This hypersensitivity was ascribed to the TRIC agent rather than to egg material in the vaccine. By contrast, no such reaction was seen in vaccinated children who subsequently acquired trachoma. Grayston (1963) also reported that pannus developed in a proportion of monkeys after conjunctival inoculation with TRIC agents. This lesion was observed only in animals previously inoculated with TRIC or psittacosis agents by the parenteral or conjunctival

routes, and was also ascribed to hypersensitivity. To the best of our knowledge, no corneal lesions developed in any of our baboons, but minor degrees of pannus might well have escaped detection under the low magnification used for the clinical examinations.

SUMMARY

A mineral oil adjuvant failed to enhance or to prolong the somewhat short-lived immunity induced in baboons by trachoma/inclusion conjunctivitis (TRIC) vaccines, and was of little or no value in increasing or prolonging the formation of group complement-fixing (CF) antibody. Vaccines prepared with protamine sulphate or with calcium phosphate were no more immunogenic than the untreated parent suspensions.

Cross tests with aqueous vaccines revealed antigenic differences in the TRIC agents examined, in terms of their ability to protect against conjunctival challenge. Strain MRC–1/OT differed from MRC–17 and from MRC–4, and MRC–187 from MRC–4 *f*.

The four Gambian trachoma agents tested were much less effective than the MRC–4 strain of inclusion conjunctivitis in inducing group CF antibody in baboons. The titre of circulating antibody bore no relation to the state of immunity to conjunctival challenge.

In three baboons given mineral oil adjuvant vaccine and in one given aqueous vaccine the conjunctival responses to subsequent challenge were significantly enhanced. This reaction may be an expression of a hypersensitive state induced by relatively ineffective vaccines; like immunity, it is unrelated to the titre of CF antibody in the serum.

We are most grateful to Mr P. Avis (Pfizer Ltd.) for his kindness in undertaking the statistical computations; to Miss Anne Smith and Miss Fay Storey for the complement fixation tests; to Dr Doris Graham for preparing the calcium phosphate vaccine; to Dr F. Himmelweit for his helpful advice concerning mineral oil adjuvants; and to the Pennysylvania Refinery Company, Pennysylvania, U.S.A., for their generous gift of Drakeol 6 VR.

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