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Studies on type C influenza virus in the chick embryo

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SUMMARY

The effect of varying conditions of inoculation and incubation on the growth of type C influenza virus in the allantoic cavity of the developing chick embryo were investigated. It was found that the highest yields of both virus haemagglutinin and infectious virus were obtained following the inoculation of chick embryos at 8 days with subsequent incubation at 32° C. Using the chick embryo allantoic cavity for titration of infectious virus, growth curves of allantoically propagated virus under varying inoculation and incubation conditions were determined.

INTRODUCTION

Although type C influenza virus is recovered only infrequently from infections in man, it appears to be widespread in nature and, since the initial isolation of the virus in 1947 from an individual with a mild respiratory tract infection (Taylor, 1949), strains of influenza type C have been recovered in many areas of the world (Fukumi, Sunakana, Takaki & Tanaka, 1951; Zhdanov, 1959; Veeraraghavan, 1961; Jennings & Grant, 1967). Antibodies to type C influenza virus are common in sera from most age groups of the population as surveys carried out in England (Andrews & MacDonald, 1955), North America (Davenport, Hennessy & Francis, 1953) and the Caribbean (Jennings, 1968) have shown.

The infrequent isolation of the virus may be due, at least in part, to the type of infection produced which is usually a sporadic, mild or asymptomatic upper respiratory tract illness, while the high incidence of type C influenza virus antibody may result from a high rate of re-infection (Zhdanov, 1959).

Detailed studies on the biological properties and laboratory behaviour of type C influenza virus may be hampered by the difficulties often encountered in propagating the virus in any system other than the amniotic cavity of the chick embryo. Several attempts have been made to cultivate the virus in laboratory animals (Taylor, 1951; Morozenko, 1957) and in tissue cultures (Green, Lieberman & Mogabgab, 1957; Korych & Frankova, 1966) but with little success. Following the intra-allantoic inoculation of chick embryos, the production of infectious virus and virus haemagglutinin by type C influenza virus has been reported as erratic and irregular (Taylor, 1951), the titres obtained often being considerably lower than those produced in the amniotic cavity.

However, more recent reports have suggested that satisfactory growth of the

virus does occur in the allantois if incubation is carried out at 32° to 33° C. for 72–84 hr. (Deichman, 1958; Styk, 1963), and use of this system may facilitate studies on some biological properties of the virus.

The present report confirms that type C influenza can be cultivated in the chick embryo allantoic cavity and determines the conditions of inoculation and incubation leading to the greatest yields of virus.

MATERIALS AND METHODS

Virus

The 1233 strain of influenza type C virus (C/1233), originally isolated in 1947 (Taylor, 1949), was obtained from the Research Reference Reagents Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, U.S.A. The virus received 13 serial passages in the amniotic cavity of 10- or 11-day-old chick embryos in our laboratory after which an amniotic fluid pool of the virus was prepared and used for studies on virus growth in the chick embryo allantoic sac. The identity of the virus was confirmed by haemagglutination-inhibition tests against influenza A, B, C and parainfluenza standard reference antisera.

Infectivity titrations

Infectious virus was assayed in either the amniotic (AEID 50) or allantoic (EID 50) cavities of the chick embryo. For titration in the amniotic cavity, serial tenfold dilutions of the virus samples to be assayed were prepared in chilled Dulbecco 'A' phosphate-buffered saline (PBS), pH 7·3, and each dilution inoculated into three 10- or 11-day-old chick embryos. The volume of inoculum used was 0·1 ml. per embryo and incubation was at 35° C. for 48 hr.

For infectivity titrations by the allantoic route, virus dilutions were prepared as described above but embryos were inoculated at 8 days and incubated subsequently at 32° C. for 72 hr. Three eggs were used per dilution and the inoculum volume was 0·2 ml. per egg.

After incubation for the appropriate length of time the embryos were chilled at 4° C., either amniotic or allantoic fluids collected and each fluid spot-tested individually for the presence of virus haemagglutinin using a 1 % suspension of chicken erythrocytes in 0·85 % saline. The 50 % end-points were calculated according to the method of Reed & Muench (1938).

Haemagglutination titrations

Virus haemagglutinin was assayed in Perspex trays by standard methods (WHO Expert Committee on Influenza, 1953), using an 0·5 % suspension of chicken erythrocytes prepared in 0·85 % saline. Virus dilutions were made in saline and all haemagglutination (HA) tests with influenza C/1233 were performed at 4° C.

RESULTS

Production of haemagglutinin by C/1233 in the chick embryo allantois

To determine optimum conditions for the growth and production of haemagglutinin by influenza type C/1233 in the chick embryo allantoic cavity, a pool of amniotically propagated virus containing 6400 haemagglutinating units per ml. (HAU/ml.) and $10^{10\cdot0}$ AEID50 per ml. was inoculated intra-allantoically, in 0·2 ml. amounts, into groups of embryos aged either 8 or 10 days and these were incubated subsequently at either 32° or 35° C. The inoculum dilutions, prepared in cold PBS, contained $10^{3\cdot0}$, $10^{4\cdot0}$ and $10^{5\cdot0}$ AEID50/0·1 ml. After incubation for 72 hr., the embryos were chilled, the allantoic fluids collected and tested individually for virus haemagglutinin.

Table 1 shows the combined results of this experiment as the concentration of virus in the inoculum had no apparent effect on the incidence or level of virus haemagglutinin. Studies in this laboratory (Prasad, 1971) have failed to demonstrate the von Magnus phenomenon with influenza C virus. A significantly greater percentage of allantoic fluids collected from embryos inoculated at 8 days, in contrast to fluids from embryos inoculated at 10 days, contained detectable virus haemagglutinin, irrespective of the temperature of incubation. However, there appeared to be some effect of incubation temperature on the production of virus haemagglutinin, and in fluids collected from embryos inoculated at 10 days virus haemagglutinin was detected most frequently after incubation at 32° as opposed to 35° C. Similarly, in allantoic fluids from embryos inoculated at 8 days, the presence of haemagglutinin was observed slightly more frequently after incubation at the lower temperature.

Table 1 also shows the percentage of the positive allantoic fluids with virus haemagglutinin titres of at least 400 HAU/ml. Such relatively high titres were commonly observed only in allantoic fluids collected from embryos inoculated at 8 days and incubated subsequently at 32° C., and 22 (45 %) of these fluids were observed to have titres of at least 400 HAU/ml. The least successful conditions for the production of influenza C/1233 haemagglutinin followed the inoculation of embryos at 10 days and incubation of these embryos at 35° C.

Table 1. *Production of virus haemagglutinin by amniotically propagated influenza C in the chick embryo allantois*

Embryo age at inoculation (days)	Incubation temperature (° C)	Number of fluids tested	Fluids positive for haemagglutinin		Positive fluids with titres ⩾ 400 HAU/ml. (%)
			No.	%	
8	32	63	49	77·9	44·9
8	35	63	45	71·4	13·7
10	32	49	32	65·4	19·1
10	35	54	17	31·7	17·6
Totals		229	143	62·3	25·8

Production of infectious virus by C/1233 in the chick embryo allantois

The production of infectious virus following the intra-allantoic inoculation of amniotically propagated influenza type C/1233 into chick embryos was examined in four separate experiments. In each of these groups embryos aged 8 or 10 days were inoculated intro-allantoically with 0·2 ml. amounts of an amniotic fluid pool of C/1233 containing $10^{4.5}$ or $10^{5.0}$ AEID 50/0·1 ml. These embryos were incubated at either 32° or 35° C. for 72 hr. After chilling, the infected allantoic fluids were collected and equal volumes pooled according to embryo age at inoculation and temperature of incubation. These pools were then titrated, intra-amniotically, for infectious virus and also tested for virus haemagglutinin.

The results of these experiments are shown in Table 2 and it can be seen that, for each experiment, the amount of infectious virus varied little under the different conditions, with the exception that the titres of infectious virus in allantoic fluid pools prepared from embryos inoculated at 8 days and incubated subsequently at 32° C. were always at least tenfold higher than the values observed in pools from embryos inoculated at 10 days or incubated at 35° C.

Serial passage of influenza C/1233 in the chick embryo allantois

Amniotically propagated influenza type C/1233 was now subjected to a series of passages in the chick embryo allantoic cavity, under those conditions which appeared from the above experiments to result in the best yields of infectious virus and virus haemagglutinin. Virus dilutions in chilled PBS were inoculated, in 0·2 ml. amounts, intra-allantoically into groups of 8-day-old embryos at each passage and these were incubated at 32° C. for 72 hr. The titres of virus haemagglutinin were determined in each allantoic fluid at each passage. At the tenth passage level an allantoic fluid pool of the virus was prepared and assayed for infectious virus, using the intra-allantoic method, and for virus haemagglutinin. This pool contained 1280 HAU/ml. and an infectivity titre of $10^{9.2}$ EID 50/ml.

Growth of allantoically propagated C/1233 in the chick embryo allantois

Two experiments to determine the growth curves produced by allantoically propagated influenza C in the chick embryo allantoic cavity under varying conditions of inoculation and incubation were now carried out. In one, growth of C/1233 in chick embryos inoculated at 8 days and incubated subsequently at either 29°, 32° or 35° C. was compared. In the second experiment, production of virus haemagglutinin and infectious virus after the inoculation of C/1233 into 6-, 8- or 10-day-old embryos incubated subsequently at 32° C. were compared.

Both experiments were performed in the same way. Chick embryos aged 6, 8 or 10 days were each inoculated with 0·2 ml. of allantoically propagated C/1233 diluted in chilled PBS to contain $10^{3.5}$ EID 50/0·2 ml., and incubated at the appropriate temperature. At intervals after inoculation, groups of four embryos were chilled, the allantoic fluids collected and equal volumes pooled. These pools were stored at −70° C. before assay for haemagglutinin and infectious virus. Infectivity titrations were carried out by the intra-allantoic method.

Table 2. *Production of infectious virus and virus haemagglutinin by amniotically propagated influenza C in the chick embryo allantois*

Experiment number	1				2				3				4		
Inoculum concentration/ 0·1 ml. ...	$10^{4.5}$ AEID 50				$10^{5.0}$ AEID 50				$10^{5.0}$ AEID 50				$10^{5.0}$ AEID 50		
Embryo age (days)	8	8	10	10	8	8	10	10	8	8	10	10	8	8	10
Incubation temp. (°C)	32	35	32	35	32	35	32	35	32	35	32	35	32	35	32
No. of fluids in pool	12	12	11	10	6	6	8	8	20	19	15	20	14	14	16
Haemagglutinin titre/ml.	200	25	200	100	160	160	160	20	800	200	50	< 5	400	50	25
AEID 50 titre/ml.	8·66	7·24	7·50	7·50	8·70	7·00	7·29	7·24	8·24	7·17	7·00	7·00	7·62	6·29	6·50

Table 3. *Growth of allantoically propagated influenza C in the allantois of 8-day-old chick embryos at various incubation temperatures*

Time after inoculation (hr.)	Incubation temperature: 29° C HA titre	29° C EID 50 titre	29° C EID 50/HA ratio	32° C HA titre	32° C EID 50 titre	32° C EID 50/HA ratio	35° C HA titre	35° C EID 50 titre	35° C EID 50/HA ratio
6	< 2	$10^{2.53}$	—	< 2	$10^{2.20}$	—	< 2	$10^{2.15}$	—
12	< 2	$10^{1.87}$	—	< 2	$10^{2.85}$	—	< 2	$10^{3.20}$	—
24	< 2	$10^{3.52}$	—	< 2	$10^{5.18}$	—	< 2	$10^{3.86}$	—
36	10*	$10^{8.19}$	7·19	40	$10^{7.51}$	5·91	< 2	$10^{6.84}$	—
48	< 2	$10^{6.85}$	—	120	$10^{7.86}$	6·78	20	$10^{7.19}$	5·89
60	80	$10^{8.52}$	6·62	320	$10^{8.52}$	5·91	10	$10^{6.86}$	5·86
72	20	$10^{6.18}$	4·88	960	$10^{8.52}$	5·54	40	$10^{7.86}$	6·26

* Haemagglutination (HA) titres expressed as the reciprocal of the dilution. Titres expressed as values per ml.

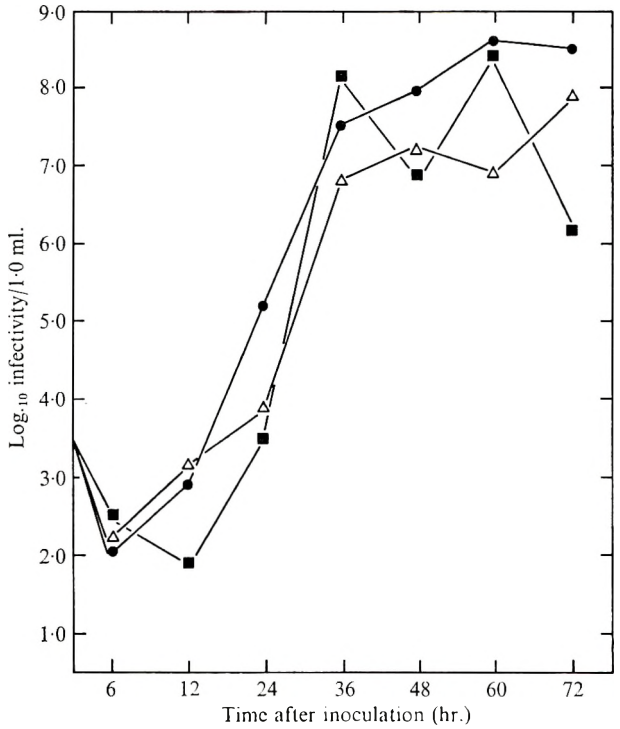

Fig. 1. Growth curves of influenza C/1233 in the allantois of 8-day-old eggs after incubation at various temperatures. ■—■, 29°; ●—●, 32°; △—△, 35°.

Fig. 1 shows the growth curves obtained following incubation at various temperatures after inoculation of virus into embryos at 8 days. Relatively high titres of infectious virus were observed at 32° C. and growth was rapid in the early stages of infection at this temperature. The amount of infectious virus present after 72 hr. at 32° C. was greater than that observed after incubation at 29° or 35° C. for the same period. The infectious virus titres at the higher temperature never reached those observed at 32° C., while at 29° C. the production of infectious virus appeared more variable.

The titres of infectious virus, together with the haemagglutinin and EID50/HA ratios, are shown in Table 3. At 48, 60 and 72 hr. after infection, the virus haemagglutinin titres in the pooled allantoic fluids from embryos incubated at 32° C. were at least fourfold greater than in pools from those incubated at 29° or 35° C. At all temperatures haemagglutinin was undetectable before 36 hr. after virus inoculation.

In Fig. 2 the growth curves obtained after the intra-allantoic inoculation of chick embryos at 6, 8 or 10 days with subsequent incubation at 32° C. are shown. The greatest amount of infectious virus was observed in allantoic fluid pools collected at 60 hr. from embryos inoculated at 8 days. The infectivity titres in pools from 6- and 10-day-old embryos approached this value at 60 hr., but in embryos inoculated at 10 days, the titre had dropped considerably by 84 hr. The production of virus haemagglutinin at 72 and 84 hr. was highest in embryos

Table 4. *Growth of allantoically propagated influenza C in the allantois of chick embryos inoculated at different ages and incubated at 32° C.*

Time after inoculation (hr.)	Age of embryo at inoculation: 6 days			8 days			10 days		
	HA titre	EID 50 titre	EID 50/HA ratio	HA titre	EID 50 titre	EID 50/HA ratio	HA titre	EID 50 titre	EID 50/HA ratio
6	< 2	$10^{1.00}$	—	< 2	$10^{1.00}$	—	< 2	$10^{1.00}$	—
12	< 2	$10^{2.52}$	—	< 2	$10^{2.52}$	—	< 2	$10^{2.52}$	—
24	< 2	$10^{2.15}$	—	< 2	$10^{3.86}$	—	< 2	$10^{2.15}$	—
36	< 2	$10^{6.85}$	—	< 2	$10^{7.15}$	—	160	$10^{7.15}$	4·94
48	< 2	$10^{6.20}$	—	10	$10^{6.53}$	5·53	< 2	$10^{6.18}$	—
60	160*	$10^{7.85}$	5·64	80	$10^{8.18}$	6·28	120	$10^{7.86}$	5·78
72	240	$10^{7.18}$	4·80	320	$10^{7.86}$	5·35	40	$10^{7.53}$	5·93
84	240	$10^{7.53}$	5·15	640	$10^{7.53}$	4·72	120	$10^{5.86}$	3·78

* Haemagglutinin (HA) titres expressed as the reciprocal of the dilution. Titres expressed as values per ml.

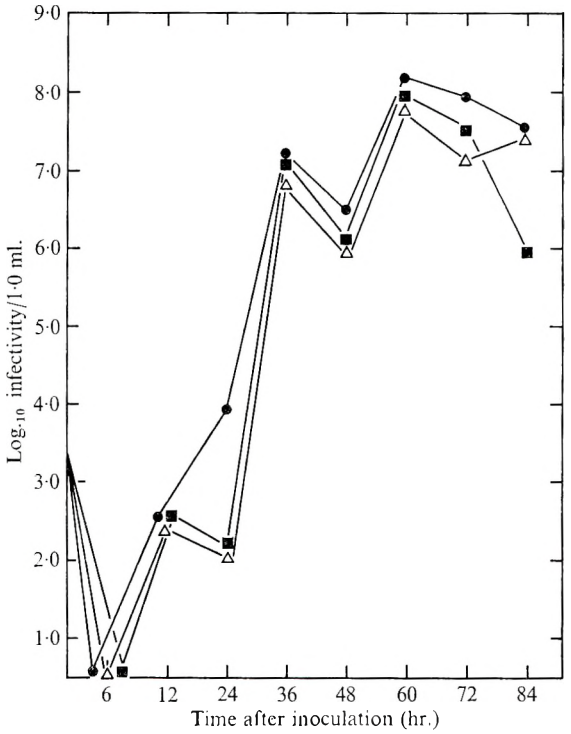

Fig. 2. Growth curves of influenza C/1233 in the allantois of 6-, 8- and 10-day-old eggs incubated at 32°. △—△, 6-day-old eggs; ●—●, 8-day-old eggs; ■—■, 10-day-old eggs.

inoculated at 8 days (Table 4), but was undetectable before 48 hr. after infection. In allantoic fluid pools from embryos infected at 6 days, virus haemagglutinin remained undetectable before 60 hr. after infection.

DISCUSSION

Although antibody studies suggest that type C influenza virus is a common pathogen of man (Davenport *et al.* 1953; Andrews & MacDonald, 1955; Jennings, 1968) it is usually associated with mild or inapparent infections (Zhdanov, 1959). It is perhaps for this reason, combined with the lack of a suitable system for cultivation which, besides consistently producing adequate quantities of virus, lends itself readily to experimentation, that type C influenza virus is rarely subjected to laboratory examination. The present work was carried out to explore the possibilities of using the chick embryo allantoic cavity for investigating some biological properties of the virus.

Taylor (1951) endeavoured to grow influenza type C in the allantois of the developing chick embryo. He reported that, in eight attempts to cultivate amniotically propagated virus after intra-allantoic inoculation, virus haemagglutinin was observed only once. Taylor also found that growth of influenza type C was inconsistent and unpredictable on serial passage in the allantoic cavity. However,

satisfactory multiplication of C/1233 in the allantoic sac of 10-day-old embryos incubated at 32° to 33° C. has been reported more recently by Styk (1963), and Cantell *et al.* (1965) observed influenza type C haemagglutinin titres of 32–256 HAU/ml. in fluids from embryos inoculated intro-allantoically at 8 days and incubated at 37° C.

The present study confirms the ability of amniotically propagated influenza C to grow satisfactorily in the chick embryo allantois, and shows that the greatest quantities of haemagglutinin and infectious virus are obtained by infecting embryos at 8 days with subsequent incubation at 32° C. Under these conditions the titres of infectious virus frequently reached $10^{8\cdot0}$ AEID 50/ml., while haemagglutinin titres above 400 HAU/ml. were commonly observed in individual allantoic fluids.

However, growth of amniotically propagated influenza C in the allantois compares unfavourably with growth in the amnion in two ways. First, the production of haemagglutinin by C/1233 in the allantois appears to be affected considerably by egg to egg variation. Thus, only 62 % of all the allantoic fluids examined, irrespective of embryo age at inoculation and incubation temperature, contained detectable virus haemagglutinin. This variation is much less after the intra-amniotic inoculation of influenza C, but it does agree with the findings of Taylor (1951). Egg to egg variation is significantly reduced, however, in embryos inoculated intra-allantoically at 8 days and incubated at 32° C. when virus haemagglutinin is found in almost 80 % of the infected allantoic fluids. Secondly, the infectivity titres observed in allantoic fluid pools prepared after the intra-allantoic inoculation of amniotically propagated influenza C are usually 10- to 100-fold lower than the titres observed in the amnion, while the titres of virus haemagglutinin in the allantois are often tenfold lower than those found in the amnion.

Nevertheless, the allantoic inoculation of chick embryos at 8 days followed by incubation at 32° C. does provide satisfactory yields of infectious virus and virus haemagglutinin in pooled allantoic fluids and may enable some biological properties of influenza type C to be examined more readily. The EID 50/HA ratios for C/1233 in the allantois compare well with values obtained after inoculation into the amnion (Isaacs & Donald, 1955).

Ten serial passages of influenza type C in the allantois of 8-day-old embryos at 32° C. failed to increase the titre of infectious virus significantly, while the production of haemagglutinin remained somewhat variable. In pooled allantoic fluids haemagglutinin titres ranging from 400 to 1600 HAU/ml. could normally be obtained. Allantoically propagated influenza type C produced less haemagglutinin and infectious virus when incubated at temperatures above or below 32° C. in embryos older or younger than 8 days. These results are in contrast to findings with the PR8 strain of influenza type A (Miller, 1944) which was best cultivated in the allantoic cavity at 36° C. and produced lower haemagglutinin titres at 33° C. Early studies on the Lee strain of influenza type B showed that the greatest amounts of haemagglutinin and infectious virus were produced at 35° C. (McLean *et al.* 1944). It is interesting to note that the age of the chick embryo at inoculation affects the growth of some parainfluenza viruses in the allantoic cavity (Hsuing &

Van de Water, 1966), but this factor appears to play little part in the replication of influenza types A and B viruses.

Another group of respiratory viruses, the rhinoviruses, when cultivated in some tissue culture systems, resemble influenza type C in growing best at 32° to 33° C. (Tyrrell & Parsons, 1960). These viruses, like influenza C, are primarily pathogens of the upper respiratory tract in man and are associated with mild infections.

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Interferon induction by influenza type C

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SUMMARY

The presence of a heat-stable interferon-like inhibitor in allantoic and amniotic fluids collected from chick embryos infected with type C influenza virus was determined. This inhibitor was characterized as an interferon and the ability of both live and ultra-violet-irradiated influenza type C virus to induce the substance was examined under various conditions.

INTRODUCTION

In a previous communication (Jennings & Freeman, 1972), the production of infectious virus and virus haemagglutinin by influenza type C/1233 in the chick embryo allantoic cavity was examined and the possibility of using this system as a means for investigating some of the biological properties of influenza C discussed.

A property of many myxoviruses and paramyxoviruses is the ability to induce the formation of interferon, a substance able to inhibit the growth of most viruses, probably by indirectly disrupting the synthesis of viral proteins (Marcus & Salb, 1966) in suitable host-cell systems. Interferon was first detected with a member of the myxovirus group, a type A influenza virus, which had been inactivated by heat-treatment (Isaacs & Lindenmann, 1957) and since then many related viruses, inactivated either by heat or by ultra-violet (UV) irradiation (Burke & Isaacs, 1958*a*; Henle, Henle, Deinhardt & Bergs, 1959; Cantell, 1961), as well as live myxoviruses and paramyxoviruses (Burke & Isaacs, 1958*b*; Youngner, Scott, Hallum & Stinebring, 1966) have been found to induce interferon formation.

The ability of type C influenza virus to induce interferon has not been examined in detail, although Cantell *et al.* (1965) were able to demonstrate the presence of an interferon-like viral inhibitor in pooled allantoic fluids from chick embryos infected with influenza C. The present study confirms the appearance of a heat-stable viral inhibitor following both the intra-allantoic and intra-amniotic inoculation of live influenza C/1233 into embryonated hens' eggs, describes the characterization of the substance as an interferon and determines the effect of UV-irradiation on the ability of the virus to induce interferon.

MATERIALS AND METHODS

Viruses

The 1233 strain of influenza type C (C/1233) was used in the present study after 21 to 30 passages in the chick embryo allantoic cavity at 32° C.

The influenza B/Lee virus used to make a working standard preparation of interferon was kindly supplied by Professor K. Cantell, State Serum Institute, Helsinki, Finland. It was propagated in the allantoic cavity of 11-day-old chick embryos, using an inoculum concentration of 10^3 or 10^4 egg infectious doses (EID50) at 37° C. for 72 hr.

Semliki Forest virus (SFV), kindly provided by Dr N. B. Finter, I.C.I. Research Laboratories, Alderly Edge, Cheshire, was used as challenge virus in the assays for interferon. It was propagated in chick embryo fibroblast (CEF) tissue cultures.

Infectivity and haemagglutination (HA) titrations

These were performed by methods described elsewhere (Jennings & Freeman, 1972). Assays for infectious virus were carried out by the intra-allantoic route, using virus dilutions prepared in chilled Dulbecco 'A' phosphate-buffered saline (PBS), pH 7·3.

Preparation of standard interferon

A working standard of interferon was prepared in 11-day-old chick embryos using B/Lee influenza virus as described by Cantell *et al.* (1965). The crude interferon was partially purified by the method of Lampson, Tytell, Nemes & Hilleman (1963), omitting the column chromatography and all subsequent steps. This partially purified material contained 1280 units of interferon/ml. when assayed by the method of Fantes (1967).

Virus inactivation checks

All samples of allantoic fluid to be tested for interferon activity were heated at 60° C. for 1 hr. in a water-bath to inactivate the virus. The absence of live virus was determined by 3 serial passages in the allantoic cavity of 8-day-old chick embryos. After incubation at 32° C. for 72 hr. at each passage the embryos were chilled and the allantoic fluids tested for virus haemagglutinin. Several samples were tested similarly, by intra-amniotic inoculation into 10- or 11-day-old embryos and incubation at 35° C. for 48 hr.

Interferon assays

Interferon assays were carried out in chick embryo cells challenged with SFV (Fantes, 1967). The cells used were filtered through gauze after trypsinization and finally suspended in modified minimum essential medium (MEM) (MacPherson & Stoker, 1962) supplemented with 5% calf serum. Samples to be tested for interferon activity were serially diluted in minimum essential medium without calf serum and each dilution mixed with an equal volume of chick embryo cell suspension containing $3{\cdot}0 \times 10^6$ cells/ml. before seeding into tubes. Three tubes were used

for each dilution. After 20–24 hr. at 37° C. cell sheets were challenged with 100 TCD50 of SFV and the results read after incubation at 37° C. for a further 48 hr. The end-point was taken as the highest dilution of the sample that completely inhibited SFV cytopathic effect. The working standard of interferon was titrated with each assay.

RESULTS

Production of an interferon-like inhibitor in the allantois of chick embryos infected with influenza C

In an attempt to detect the presence of interferon in allantoic fluids from chick embryos infected with influenza C/1233 at different ages and subsequently incubated at different temperatures, groups of ten embryos were inoculated with $10^{3\cdot0}$ EID50 of the virus prepared in PBS. Each embryo was inoculated intra-allantoically with 0·2 ml. of virus dilution and two groups, one of 8-day-old, the other of 10-day-old chick embryos, were incubated at 32° C. and the remaining two groups, similarly 8- and 10-day-old, incubated at 35° C.

After 72 hr. eggs were chilled, the allantoic fluids harvested and equal volumes pooled. Four pools, corresponding to the groups inoculated, were prepared and tested for haemagglutinin, infectivity and, after heating at 60° C. for 1 hr., for interferon. A substance able to inhibit the appearance of SFV cytopathic effect in chick embryo tissue culture was detected in three pools (Table 1) but was not observed in the pool prepared from chick embryos inoculated at 10 days and incubated at 35° C. No such inhibition was produced by heat-treated allantoic fluids from uninfected chick embryos tested at similar dilutions in the same way.

The highest titre of this material was found in fluids from eggs infected at 8 days and incubated subsequently at 32° C., and this pool also contained the greatest amount of virus as determined by both HA and infectivity titrations, and there appeared to be a positive correlation between the yield of C/1233 virus and the titre of the interferon-like inhibitor.

This correlation was examined further as shown in Table 2 which includes the results of three separate experiments. In each experiment groups of 8-day-old chick embryos were inoculated intra-allantoically with C/1233 virus diluted in PBS. Each embryo was inoculated with 0·2 ml. of virus dilution and, after incubation for 72 hr. at either 32° or 35° C., embryos were chilled, individual allantoic

Table 1. *The production of a viral inhibitor in the allantois of chick embryos infected with influenza C*

Embryo age at inoculation	Incubation temperature (°C)	Haemagglutinin titre	Infectivity (titre/ml.)	Inhibitor titre
8 days	32	1600	$10^{8\cdot70}$	80
	35	400	$10^{7\cdot35}$	20
10 days	32	400	$10^{7\cdot94}$	40
	35	25	$10^{5\cdot99}$	<10

All titres expressed as reciprocals of the end-point dilution.

fluids harvested and tested for virus haemagglutinin. Fluids with relatively high haemagglutinin titres, indicating good virus growth, were pooled in equal volumes and similar pools prepared from fluids with low haemagglutinin titres. Fluids of intermediate haemagglutinin titre were not included in the pools. Samples of each pool were stored at −70° C. and subsequently titrated for virus haemagglutinin, infectivity and viral inhibitor.

The results show that those pools with high haemagglutinin and EID 50 titres also gave good yields of the interferon-like inhibitor and in fact this substance was not detected in pools containing relatively small amounts of C/1233 virus. This was observed after incubation at either 32° or 35° C.

Production of an interferon-like inhibitor in the amnion of the chick embryo

To determine if an interferon-like inhibitor could be induced by influenza C/1233 in the amniotic sac of the chick embryo, two groups of 10-day-old embryos were inoculated intra-amniotically with 0·1 ml. amounts of $10^{4.5}$ EID 50 of allantoically propagated virus. After incubation for 48 hr. at either 32° or 35° C., the embryos were chilled, the amniotic fluids collected and pooled. Eight-day-old embryos, inoculated intra-allantoically with the same virus dose and incubated at similar temperatures for 72 hr. were set up for comparative purposes, and used to prepare allantoic fluid pools.

Table 2. *Relationship between the growth of influenza C and viral inhibitor in the allantois of the chick embryo*

Incubation temperature	32° C.						35° C.			
Virus dose inoculated (EID 50)	$10^{3.0}$		$10^{3.5}$		$10^{4.5}$		$10^{3.0}$		$10^{3.5}$	
No. of embryos inoculated	10		29		37		10		29	
No. of fluids in pool	3	3	5	6	8	8	2	3	5	12
Haemagglutinin titre	3200	10	1200	10	1600	25	2400	10	800	5
Infectivity titre	$10^{8.9}$	$10^{6.7}$	$10^{7.0}$	$10^{6.0}$	$10^{7.1}$	$10^{7.2}$	$10^{7.2}$	$10^{5.7}$	$10^{7.2}$	$10^{5.7}$
Inhibitor titre	20	<10	20	<5	20	<5	80	<10	5	<5

All titres expressed as reciprocals of the end-point dilution.

Table 3. *Production of a viral inhibitor in the chick embryo amniotic cavity*

Site of inoculation	Amnion		Allantois	
Embryo age (days)	10	10	8	8
Incubation time (hr.)	48	48	72	72
Incubation temperature (°C.)	32	35	32	35
Haemagglutinin titre	3200	1600	400	200
Infectivity titre/ml.	$10^{8.9}$	$10^{8.1}$	$10^{8.0}$	$10^{7.3}$
Inhibitor titre	64	16	8	<4

All titres expressed as the reciprocal of the end-point dilution.

All the pools were tested for infectious virus, virus haemagglutinins and viral inhibitor and the results are shown in Table 3. A substance able to inhibit the appearance of SFV cytopathic effect in chick embryo tissue cultures was detected in both heat-inactivated allantoic and amniotic fluid pools. The titre of this substance was significantly greater in pools prepared from infected amniotic fluids as compared to infected allantoic fluids.

Characterization of the interferon-like inhibitor induced by C/1233

The heat-stable, virus-inhibiting substance present in influenza C-infected allantoic fluids was characterized as an interferon in the following manner. Samples of a pool of allantoic fluid containing the inhibitor, after low-speed centrifugation and heating at 60° C. for 1 hr., were treated in various ways including dialysis against 0·1 M glycine/HCl buffer (pH 2·1) for 24 hr. at 4° C.; reaction to a final concentration of 0·5 mg./ml. of crystalline trypsin for 1 hr. at 37° C.; treatment with specific influenza C/1233 antiserum and centrifugation at 150,000 *g* for 1·5 hr. Only treatment with trypsin, which reduced the titre of the inhibitor to an undetectable level, had any significant effect.

As a further test, a sample of inhibitor-containing allantoic fluid was subjected to ultracentrifugation at 100,000 *g* for 1·5 hr. and the resulting supernatant fluid and deposit (after resuspension in PBS) assayed for infectious virus and interferon. The results are shown in Table 4 and it can be seen that the deposit from the ultracentrifugation, although containing a considerable amount of infectious virus, contained no detectable interferon, all of which was recovered in the supernatant.

In addition, the crude, heated, inhibitor-containing allantoic fluid, although able to protect chick embryo cell monolayers against challenge by 100 TCD50 of SFV, failed to protect primary calf kidney cell monolayers against a similar dose of the same virus.

Temporal relationship between virus growth and interferon production

The relationship between the production of infectious virus, virus haemagglutinin and interferon with time was examined by inoculating 8-day-old chick embryos intra-allantoically with $10^{3\cdot5}$ EID50 of virus diluted in chilled PBS. Each embryo was inoculated with 0·2 ml. of virus dilution and incubated at 32° C. for 72 hr. Groups of five embryos were chilled at intervals, the allantoic fluids collected, pooled in equal volumes and stored at − 70° C. prior to assay for virus and interferon.

Table 4. *Lack of relationship between viral inhibitor and infectious virus particles*

Material tested	Titre of viral inhibitor	Titre of infectious virus
Original	8	$10^{8\cdot7}$
Supernatant from ultracentrifugation	8	$10^{4\cdot0}$
Resuspended deposit from ultracentrifugation	<2	$10^{6\cdot0}$

Inhibitor titre expressed as the reciprocal of the end-point dilution.

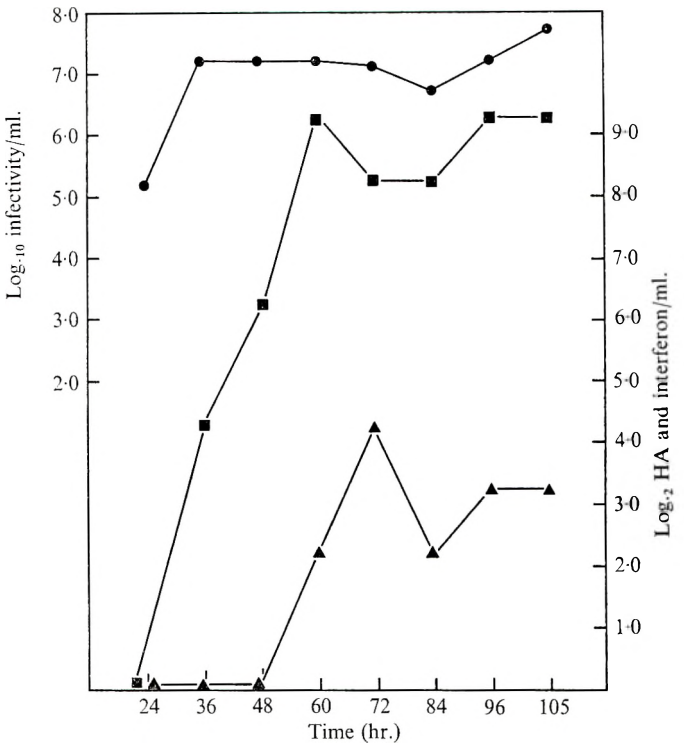

Fig. 1. Relationship between the production of infectious virus, virus haemagglutinin and interferon by C/1233 in the chick embryo allantois. ●—●, Infectious virus. ■—■, Virus haemagglutinin. ▲—▲, Interferon.

The results of these assays can be seen in Fig. 1. Interferon did not become detectable in allantoic fluid pools until 60 hr. after virus infection, and appeared considerably later than both infectious virus and virus haemagglutinin. The maximum amount of interferon, 20 units/ml., was observed at 72 hr. after infection.

Effect of ultra-violet light on the interferon-inducing capacity of influenza C

To determine whether influenza C/1233 virus was able to induce the formation of interferon following UV-irradiation, a pool of C/1233-infected allantoic fluid with an infectivity titre of $10^{8\cdot2}$ EID 50/ml. and containing 1280 haemagglutinating units/ml. was clarified by centrifugation at 2000 rev./min. for 10 min., and then dialysed against PBS, pH 7·3, for 48 hr. at 4° C. Samples of the resulting material were subjected to irradiation with UV-light for varying periods of time up to 2 min., in 5 cm. Petri-dishes 7 cm. away from the light source, a Hanovia 'Chromatolite' lamp with maximum emission at 2537 Å. The approximate thickness of the 3 ml. samples under irradiation was 1·5 mm. and they were gently agitated during exposure.

Assays for infectious virus and virus haemagglutinin were performed at all stages of the experiment and the irradiated samples tested for their ability to induce interferon. Each sample was diluted 1/100 in chilled PBS and inoculated, in 0·2 ml. amounts, intra-allantoically into groups of 8-day-old chick embryos. After incuba-

tion at 32° C. for 72 hr. the allantoic fluids were collected, pooled and assayed for interferon.

The results are shown in Table 5. No detectable interferon was induced by influenza C/1233 after UV-irradiation under these conditions although the non-irradiated control virus after clarification and dialysis, procedures which resulted in a considerable decrease in the amount of infectious virus and haemagglutinin, was able to do so.

Further experiments to induce interferon with irradiated influenza C, using both higher and lower inoculum concentrations and samples exposed to UV-light for shorter and longer periods, failed to elicit the appearance of interferon in the chick embryo allantoic cavity.

DISCUSSION

The ability of live influenza virus to induce the formation of interferon in cells of the chick embryo chorioallantoic membrane was first demonstrated by Burke & Isaacs (1958*b*), using influenza A/Melbourne. The induction of interferon by a type B influenza virus, B/Lee, in the chick embryo allantois was reported subsequently (Hahnemann & Reinicke, 1965) and since then the appearance of interferon in the allantoic fluid of chick embryos inoculated with live influenza viruses has been observed by many workers (Wagner, 1961; Vaczi, Hadhazy & Horvath, 1963; Cantell *et al.* 1965; Fantes, 1967). Most of these studies were carried out with influenza type A or B viruses, but Cantell *et al.* (1965) did find low levels of an interferon-like inhibitor in allantoic fluids from chick embryos infected with influenza type C/1233. Earlier observations (Hirst, 1950) showed that live C/1233 virus could interfere with the growth of both Newcastle disease virus in the chick embryo allantois and Western equine encephalitis virus in the mouse brain.

The present report demonstrates the appearance of a heat-stable inhibitor, able to prevent the cytopathic effect produced by SFV in CEF tissue cultures, in both allantoic and amniotic fluid pools collected from embryos infected with influenza C/1233 virus. This viral inhibitor, subsequently characterized as an interferon, could be detected after the incubation of infected chick embryos at either 32° or

Table 5. *Effect of UV-irradiation on the interferon-inducing ability of influenza C*

Treatment	Titre of infectious virus/ml.	Haemagglutinin titre	Titre of induced interferon
Centrifugation, 2000 r.p.m. for 10 min.	$10^{6\cdot2}$	50	NT
Dialysis × PBS for 48 hr. at 4° C.	$10^{5\cdot2}$	<10	5
UV-irradiation for 10 sec.	$10^{4\cdot0}$	<10	<2
UV-irradiation for 20 sec.	$10^{3\cdot7}$	<10	<2
UV-irradiation for 40 sec.	$10^{2\cdot5}$	<10	<2
UV-irradiation for 80 sec.	$<10^{1\cdot0}$	<10	<2
UV-irradiation for 120 sec.	$<10^{1\cdot0}$	<10	<2

All titres expressed as the reciprocal of the end-point dilution. NT = Not tested.

35° C. In both allantoic and amniotic fluid pools, however, the titre of the inhibitor appeared to be highest at the lower temperature, and it is at this incubation temperature, 32° C., at least in the chick embryo allantois, that the greatest yields of influenza type C/1233 are produced (Deichman, 1958; Jennings & Freeman, 1972).

This finding is in apparent contrast to that of other workers (Vaczi *et al.* 1963), who observed the induction of interferon by influenza A/PR8 to be greatest at temperatures higher than the optimum growth temperatures of the virus. However, by preparing pools from chick allantoic fluids containing relatively large amounts of virus it was possible to demonstrate high interferon titres induced by influenza C after incubation at 35° C., whereas, after incubation at 32° C., allantoic fluid pools with low virus titres contained no detectable interferon. Interferon induction by live influenza C virus thus depends, in part, on the amount of virus replication, which is greatest, and affected least by egg to egg variations (Jennings & Freeman, 1972), at 32° C.

The viral inhibitor induced by influenza C/1233 in the chick embryo allantois possesses many properties of an interferon. It is non-sedimentable, non-dialysable and unaffected by acid pH and by heat at 60° C. for 1 hr., but is destroyed by trypsin. It is not active in primary calf kidney tissue cultures and is not identical with infectious virus particles. Interferon induced by influenza C is only detected in chick embryo allantoic fluids at a late stage of virus growth, after the appearance of infectious virus and virus haemagglutinin and this is similar to interferon formation following the infection of chick embryos with live influenza A or B viruses (Wagner, 1961; Vaczi *et al.* 1963; Cantell *et al.* 1965; Hahnemann & Reinicke, 1965).

Some myxoviruses and paramyxoviruses, after inactivation either by heat or by UV-irradiation, are able to induce good yields of interferon in chick chorioallantoic membranes (Burke & Isaacs, 1958*a*, *b*). Type C influenza virus on the other hand failed to induce any detectable interferon after inoculation into the chick embryo allantoic cavity after UV-irradiation. Failure of irradiated influenza C/1233 to induce interferon was observed when the virus infectivity was either partially or completely destroyed by the UV treatment. The reason for the inability of UV-irradiated C/1233 to induce interferon is unknown, but the 'interferon-inducing factor' in the virus may be highly sensitive, and hence rapidly destroyed by UV-light, or else may fail to reach a site in the cell where it can exert its effect.

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Comparison of Tanapox virus and Yaba-like viruses causing epidemic disease in monkeys

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SUMMARY

The virus of Tanapox isolated from the lesions of patients during an outbreak of mild disease in Africa has been found to be indistinguishable in its biological and serological properties from a virus isolated from outbreaks of a pox virus infection in monkeys in primate centres in America. The natural hosts of this virus are believed to be African monkeys and 'Tanapox virus' is proposed as a suitable designation for the virus.

INTRODUCTION

Tanapox is a mild human infection with one or sometimes two pock-like lesions in the skin, observed only in the Tana River valley of Kenya in 1957 and 1962 and fully described by Downie *et al.* (1971). The causative agent is undoubtedly a member of the pox virus group in general biological characters and electron-microscopic appearance, but has no serological relationship to other pox viruses pathogenic for man. In the laboratory Tanapox virus grows only in cultures of tissue derived from man or monkey and produces experimental infection only in monkeys. Because of these biological peculiarities of the virus and on general epidemiological grounds it was thought that Tanapox virus had a monkey reservoir from which the natives of the Tana River valley were occasionally infected. However, it has not been possible to prove this by examination of wild monkeys in the area. Thus it was of especial interest when in 1965 and 1966 there occurred outbreaks of pox virus infection in monkey colonies in California (España, 1971), in Oregon (Hall & McNulty, 1967; Nicholas & McNulty, 1968) and in Texas (Casey, Woodruff & Butcher, 1967; Crandell, Casey & Brumlow, 1969), particularly when the infection spread to men in contact with these animals with clinical and histological features very similar to natural Tanapox infection. The disease in monkeys in California was labelled Yaba-like Disease (Y.L.D.) because of its clinical similarity to the pox virus infection observed in rhesus monkeys kept at a research station in Africa (Bearcroft & Jamieson, 1958; Niven *et al.* 1961). Moreover, the virus from the monkey outbreaks in U.S.A. was shown to have the same restricted pathogenicity for animals and cytopathic effects on tissue culture as Tanapox virus (España, 1966; Hull, 1968; Schmidt, 1970) and was serologically unrelated to

vaccinia. It was now of obvious importance to determine if these Yaba-like agents were related to Tanapox virus so that the monkey reservoir hypothesis could be tested. The present paper presents the results of reciprocal studies of the viruses with antisera derived from monkeys with natural infection or deliberately immunized or artificially infected with the two agents. The results show that the two viruses are immunologically identical, and support our view that Tanapox of man is essentially a zoonosis.

MATERIALS AND METHODS

Virus strains

The Tanapox virus strain isolated in 1962 had been passed three times in tissue culture of human thyroid, four times in human amnion, nine times in W.I. cells (a continuous line derived from human embryo lung) and three times in Vero cells before being used in the tests described below. The strain of Y.L.D. virus was received by A.W.D. in April 1969 and had been passed 29 times in vervet monkey kidney cell cultures and three times in BSC-1 cells. In this paper it is referred to as the 'California' strain. The strain of '1211' was received from Dr McNulty of the Oregon Primate Centre in June 1969 and is referred to below as the 'Oregon' strain.

For much of the work detailed below, virus suspensions to be used in neutralization tests were prepared from bottle cultures of Vero or BSC-1 cells maintained in 199 medium containing 1 % foetal calf serum or in Eagle's MEM or L-15 medium containing 2 % inactivated foetal calf serum. For the immunization of monkeys, however, the virus was grown in the same tissue cultures with 1 % vervet monkey serum in place of calf serum. When the cell sheets showed lesions throughout (after 8–12 days incubation at 35° C.) the cells were scraped into the medium and the suspension spun at 2000 rev./min. for 20 min. The supernatant was discarded, the cell deposit suspended in 10 ml. of buffered distilled water and sonicated for 40 sec. The suspension was then centrifuged at 2000 rev./min. for 15 min. and the supernatant kept. Two more extractions of the sonicated deposit were made in the same way. The three extracts, containing most of the virus, were then spun in a refrigerated Spinco angle centrifuge for 30 min. at 15,000 rev./min. The supernatant fluid from the first extract was kept as antigen to be used in complement fixation (C.F.) and precipitation tests and the deposited virus from the three extracts was suspended in buffered distilled water, pooled and kept frozen at −70° C. as stock virus suspensions. For immunization of monkeys virus from Vero cells grown in vervet serum + '199' was prepared in the same way. But the crude virus suspension prepared as described was then spun through 35 % sucrose and the virus deposit washed once by high speed centrifugation in buffered distilled water and the deposit resuspended and kept for immunizing monkeys by intravenous injection.

Preparation of antisera

Sera were collected from monkeys affected by the outbreak of disease in the Primate Centre in California and at different periods after experimental infection of these animals with the California virus. In Liverpool four animals were immun-

ized with the three strains of virus; one rhesus and one vervet monkey with Tanapox virus, one vervet with Oregon virus and one rhesus with California virus. These animals were bled before immunization (serum 1). They were then infected intradermally with 0·1 ml. of stock virus suspension at each of 12 sites. Three weeks later when the lesions resulting from the injections had disappeared the animals were bled (serum 2) and given an intravenous injection of 2 ml. of the appropriate purified virus suspension. These intravenous injections were repeated 6 and 12 days later and the animals bled (serum 3) 6 days after the third injection.

Complement-fixation tests

In Liverpool the antigen was prepared from the extract of infected sonicated tissue culture cells as described above. In the Primate Centre in California the viruses were grown in bottle cultures of BSC-1 or vervet monkey kidney cells. After 6–8 days when most of the cell sheets showed specific lesions the maintenance medium was replaced by Earle's balanced salt solution with 0·5 % lactalbumin hydrolysate. The cells were scraped into this, the suspension was frozen and thawed three times and the cell debris removed by centrifugation at 5000 rev./min. for 20 min. The supernatant constituted the antigen. Residual virus was inactivated by incubation at 37° C. for 2 hr. after the addition of Beta-propiolactone to make 0·025 %. Control antigens were prepared in the same way from uninfected tissue cultures. In both laboratories antigens were titrated in doubling dilutions against doubling dilutions of antisera. The dilution chosen for further tests was the highest that gave maximum serum titre. The technique of the tests was similar in both laboratories. Mixtures of serum dilution, complement (2–2½ MHD) and antigen, 0·1 ml. of each, were held at 4° C. overnight and next morning kept at 37° C. for ½ hr. after adding the haemolytic system (España & Hammon, 1948; Downie *et al.* 1971).

Neutralization tests

All sera were inactivated before testing. Equal volumes of serum dilutions were mixed with virus suspension diluted to contain 50–100 f.f.u. (focus forming units) in 0·1 ml. and incubated at 37° C. for 2 hr.; 0·2 ml. of each mixture was then inoculated into each of 3–5 tissue culture tubes of Vero or BSC-1 cells and 1·0 ml. of maintenance medium added to each tube. A mixture of virus with dilutions of antibody-free serum was included in each test to provide the base line lesion count. After incubation of the culture tubes for 7–12 days the lesions in each tube were counted using a × 10 binocular microscope. (The tubes were examined daily from the fifth day onwards and the final count made when foci were well developed and a further daily increase in the number of foci in control tubes had ceased.) From the results the dilution of serum effecting a 50 % reduction in the mean count was estimated. In the Primate Centre in California the antibody content of sera was sometimes estimated by titrating tenfold dilutions of virus in the presence of a constant dilution of serum. From the results the neutralization index was determined by subtracting the logarithm of the virus titre in the test serum from the logarithm of the control virus titre.

Precipitation tests

These were made in 1·0% or 0·85% agar in layers 1·0 mm. thick on ordinary microscope slides. The wells in the agar layer were 4 mm. in diameter with the centres of neighbouring wells 5·5 mm. apart. Sera and antigens in neighbouring wells were allowed to react for 24 or 48 hr. at room temperature before the results were read. In some instances sera and antigens were concentrated by freeze drying and re-solution in one-fifth of the original volume of distilled water to intensify the reactions. Treatment of the agar with 1·0% tannic acid solution (Alpert, Monroe & Schur, 1970) served to accentuate lines of precipitation before photography.

RESULTS

Active immunity experiments

It had been noted in the original outbreaks of infection in the American Primate Centres that recovered animals were immune to re-infection by virus inoculated intradermally. Three monkeys that had been immunized in Liverpool by intravenous injections of virus for the preparation of antisera were tested 2 days after their final bleeding along with a normal control vervet monkey by injecting each of the three virus strains intradermally into each animal; the titre of the virus suspensions used was approximately 10^7 f.f.u. per ml. The animals were sedated on the 4th and 7th days after injection for careful examination of their lesions.

The control vervet monkey after 4 days showed raised thickened lesions about 1·5 cm. in diameter at the sites of injection of all three viruses. After 7 days the lesions had not increased much in size but showed central necrosis. The lesions thereafter regressed and showed only slight residual scabbing after 14 days. These lesions were similar in size and course of evolution to those exhibited by the monkeys when first infected intradermally with Oregon and California viruses. The vervet monkey used to prepare antisera against Tana virus had shown only small papular lesions when given its first intradermal injections of Tana virus. It was subsequently discovered that this monkey had a significant titre of neutralizing antibody to Tanapox virus before injection.

All three immunized monkeys after challenge showed only tiny papular lesions after 4 days and these had practically gone by the 7th day. Although the vervet monkey immunized with Tana virus apparently had some degree of immunity before being used in these experiments, the results indicate that the three viruses could not be differentiated by these tests.

Complement-fixation tests

The sera of monkeys which had recovered from contact or experimental infection with California virus in the Primate Centre were tested against antigens prepared from Tana and California infected tissue cultures. The results are shown in Tables 1 and 2. The titres shown in Table 2 represent the average from six monkeys which had been inoculated intradermally with a suspension of California virus prepared from the 30th serial passage in vervet monkey kidney cell cultures. The findings

recorded in Tables 1 and 2 strongly suggest that the complement-fixing antigens of Tanapox and California viruses are identical.

The sera collected before (1), during (2) and after (3) immunization of monkeys in Liverpool with the three viruses were similarly tested against tissue culture antigens. The results are shown in Table 3.

The Oregon antigen and Oregon antisera would appear to be weaker than those of the other two viruses and the Tanapox antiserum had a somewhat higher titre

Table 1. *Complement-fixing titres against California and Tana tissue culture antigens of sera from* Macaca mulatta *with spontaneous Yaba-like dise*ase

Monkey sera (*M. mulatta*)	C.F. titre of sera *vs.* antigens	
	Tanapox	California
3201	64*	64
3206	8	16
3211	64	128
3213	32	32
3216	16	32
3223	128	128
3226	32	32
3228	16	16
3242	32	64
3243	16	16
3246	128	128
3252	16	16
3257	16	16
3281	64	64
3292	128	128
3515	4	8
3693	64	32
3986	32	64
4090	16	16
4209	16	32

* Reciprocal of highest serum dilution giving a 2+ or better fixation of complement.

Table 2. *Complement-fixing titres against California and Tanapox tissue culture antigens of sera from* M. mulatta *inoculated with California virus*

Days after inoculation	C.F. titre of sera *vs.* antigens	
	California	Tanapox
0	0*	0
13	8	4
26	32	32
39	128	64
53	64	64
67	32	32
82	16	16
95	16	16
116	8	8
124	8	8

* Reciprocal of serum dilutions. Average titre of six monkeys.

than the California antiserum. However, the results indicate that there is no qualitative difference in the antigens from the three viruses.

Neutralization tests

Sera from *Macaca mulatta* with spontaneous Y.L.D. lesions which were beginning to regress neutralized the cytopathic effect induced by either Tanapox or California viruses. Table 4 shows that the neutralizing indices of four Y.L.D. monkey convalescent sera were similar against either virus.

M. mulatta inoculated intradermally with the California virus developed neutralizing antibodies within 7–10 days after inoculation. The antibody levels increased as the lesions developed, reaching a maximum between 18 and 22 days. Titres remained at this level until the lesions regressed. After regression, neutralizing antibodies decreased sharply and were barely detectable six months after inoculation. The results of representative experiments in which sera from serial bleedings of two infected monkeys were tested for their neutralizing activity against both California and Tanapox viruses are shown in Table 5.

The results of the studies presented in Tables 4 and 5 indicated that, as in the case of complement-fixation tests, Tanapox and California viruses cannot be distinguished by neutralization tests. The sera from monkeys, convalescent from and immunized against California virus infection, neutralized both viruses to a high

Table 3. *Complement-fixing titre of sera from three monkeys immunized with Tanapox, California and Oregon viruses*

	Titre of sera against antigens of		
Monkey sera	Tanapox	Oregon	California
Tanapox 1	< 10	< 10	< 10
2	40	40	40
3	160	40	160
Oregon 1	< 10	< 10	< 10
2	10	10	10
3	40	20	40
California 1	< 10	< 10	< 10
2	40	40	40
3	80	40	80

Table 4. *Neutralizing indices of Y.L.D. convalescent monkey sera* (M. mulatta) *against California and Tanapox viruses*

	Infeetivity titre of virus		Neutralization index	
Monkey serum (1/20)	California	Tanapox	California	Tanapox
982	1·3	1·0	4·3	5·0
1269	1·6	1·5	4·0	4·5
1270	2·0	2·0	3·6	4·0
1285	2·2	2·5	3·4	3·5
Normal monkey	5·0	5·3	0·6	0·7
None	5·6	6·0	—	—

titre and the neutralization index was essentially the same for both Tanapox and Y.L.D. viruses.

The sera of monkeys immunized against the three viruses in Liverpool were tested in threefold dilutions against all three viruses: the results are shown in Table 6. These tests show that all three sera neutralize all three viruses to high titre. The slight difference in titre with individual sera and viruses does not appear to be significant. The pre-immunization serum of the Tanapox monkey had low titre antibody to all three viruses and as noted above this may have determined the smallness of the lesions produced in this animal by the first intradermal injections of Tanapox virus.

Table 5. *Neutralizing activity against California and Tanapox viruses of sera from two* M. mulatta *inoculated with California virus*

		% reduction of lesion count compared with control			
		Monkey no. 3203		Monkey no. 3247	
Days after inoculation	Serum diln.	California virus	Tanapox virus	California virus	Tanapox virus
1	1/10	3·0	3·1	6·0	6·1
7	1/10	14·8	24·3	26·5	24·3
	1/40	8·8	12·2	8·8	6·1
19	1/10	91·2	87·9	73·6	75·8
	1/40	86·8	84·9	47·1	42·5
	1/160	73·6	69·7	N.T.	N.T.
44	1/10	97·0	95·5	86·8	87·9
	1/40	86·8	87·9	58·9	60·7
	1/160	75·0	74·3	50·0	51·6
183	1/10	26·5	12·2	3·0	3·1
No. of f.f.u. in virus controls		68	66		

Table 6. *Neutralization tests against Tanapox, California and Oregon viruses with the sera of monkeys immunized against the three viruses*

	Neutralizing titres of sera against		
Monkey sera	Tanapox virus	Oregon virus	California virus
Tanapox 1	60	60	30
2	600	135*	135*
3	6000	6000	6000
Oregon 1	< 5	< 5	< 5
2	500	135*	135*
3	4000	2500	2000
California 1	< 5	< 5	< 5
2	200	200	100
3	4000	2500	3000

* = Not tested at higher serum dilutions.

Precipitation tests

For these tests the third sera from the monkeys immunized against Tanapox, Oregon and California viruses were used. These sera did not produce very strong lines of precipitation when tested against antigen prepared from tissue culture infected with the three viruses. Concentration of antigen and antisera by freeze drying enhanced the reactions. An immune rabbit serum (1211), kindly supplied by Dr McNulty, had been prepared by immunization of a rabbit with Oregon virus which had been purified by sucrose density gradient centrifugation. The precipitation reactions between these immune sera with each of the three virus antigens are shown in Pl. 1, fig. 1. Allowing for differences in the precipitating potency of the sera and antigens the results seem to show reactions of identity with all three antigens tested against the four antisera.

In Davis, tests were carried out with immune animal sera (mouse, monkey, guinea pig) against Tanapox and California viruses. All sera tested reacted in the same manner against either antigen. There were no differences in the line patterns produced by either California or Tana antigens, indicating identical antigenic make-up in the two viruses.

In tests illustrated by Pl. 1, fig. 2 the position of the reagents was reversed and each antigen was tested against the four antisera placed in the surrounding cups. In this test the California and Oregon antisera and the California antigen show weak reactivity but again there seems to be identity of reactions between each antigen and the three immune monkey sera. The precipitation lines resulting from the interaction of the antigens with the immune rabbit serum '1211' do not show identity with the reactions shown by the immune monkey sera. This seems especially clear of the reactions with Oregon antigen. The immune rabbit serum did not precipitate with the control antigen from uninfected tissue culture, but this control antigen was not concentrated as were the virus antigens. The lack of identity of reactions with the immune rabbit serum when compared with those given by the immune monkey sera suggests that there were antibodies in the immune rabbit serum which were not present in the immune monkey sera. These antibodies may have been directed against antigens in normal monkey tissue cells. The observation that this immune rabbit serum fixed complement to a considerable titre with 'antigen' from uninfected tissue culture cells support this suggestion. However, the precipitation reactions between the immune monkey sera and the three virus antigens again indicate that with these reagents the antigens of the three viruses are identical.

DISCUSSION

The viruses isolated from Tanapox in man, from the natural epizootic among monkeys in the U.S.A. and from human contacts infected from them have been shown to have similar biological properties (Downie *et al.* 1971; España, 1966; Hull, 1968; McNulty *et al.* 1968). The lesions of Tanapox and those in naturally infected monkeys were histologically identical and recent electron-microscopic studies have shown that the changes produced in infected tissue culture cells by

Tanapox and California viruses are the same (España, Brayton & Ruebner, 1971). Because of the similarity of the clinical disease caused by Tanapox virus and by the virus from the epizootics in monkeys it seemed reasonable to find out whether the same virus was responsible. The results reported in this paper have shown that the viruses are identical by the various serological tests used. Nicholas has also found that an antiserum prepared in rabbits against Oregon virus neutralized Tanapox and Oregon viruses to the same titre (personal communication). The name 'OrTeCapox virus' has been suggested for the monkey virus studied in laboratories in Oregon, Texas and California (Nicholas, 1970). However, as this virus is the same as Tanapox virus, which was isolated from African patients in 1962 and recorded in 1963 (Allison, 1965), it would appear that 'Tanapox' virus might be the more appropriate designation for this member of the pox group.

The demonstration that the same virus seems to have been responsible for the outbreaks of infection among macaque monkeys in America and the epidemics of Tanapox in Kenyans has an important bearing on the epidemiology of the disease in the Tana River valley. The infected monkeys in the Primate Centres in America had been supplied by the same importer on whose overcrowded premises African and Asiatic monkeys had been housed in the same building. Because the disease affected mostly Asiatic monkeys of the genus *macaca* while African green monkeys were apparently insusceptible, it has been suggested that the virus may have spread to the macaques from inapparent infection in the African monkeys (Schmidt, 1970). The outbreaks of Tanapox observed in 1957 and 1962 occurred at times of severe flooding when the human population together with their domestic and wild animals were crowded on small islands among the flood waters (Downie *et al.* 1971). It seems likely that the human population acquired their infection from wild monkeys – in other words the native population in the Tana River valley, like the Asiatic monkeys in the Primate Centres in America, served as the indicators of latent infection with Tanapox virus in African monkeys.

We are indebted to Dr Brian Pratt for the preparation of Figs. 1 and 2, and to Mrs Moruzzi for skilful technical assistance. The work in Liverpool was supported by a grant from the Medical Research Council.

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EXPLANATION OF PLATE

Rabbit serum '1211' was prepared against purified Oregon virus. Tan = Tanapox; Ore = Oregon; Cal = California; Con = control; A = antigen; S = serum.

Fig. 1. Precipitation test in agar gel in which each of four antisera were tested against antigens of Tanapox, Oregon and California viruses.

Fig. 2. Precipitation test in agar gel in which four antigens were tested against Tanapox, Oregon, California and control antisera.

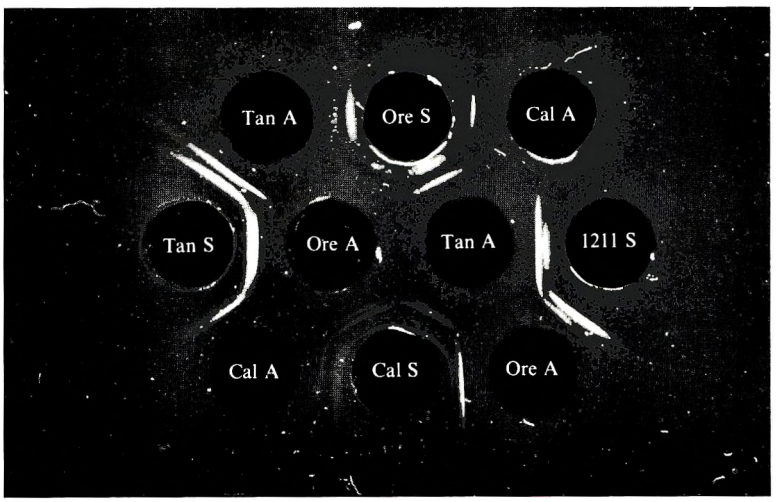
Tan A
Ore S
Cal A
Tan S
Ore A
Tan A
1211 S
Cal A
Cal S
Ore A

Fig. 1.

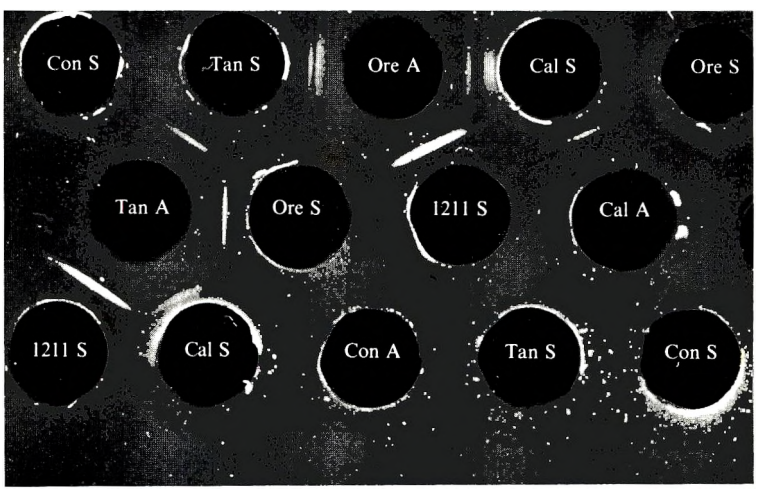
Con S
Tan S
Ore A
Cal S
Ore S
Tan A
Ore S
1211 S
Cal A
1211 S
Cal S
Con A
Tan S
Con S

Fig. 2.

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Some consequences of the multiple infection of cell cultures by TRIC organisms

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SUMMARY

In BHK 21 cells infected with more than one TRIC organism the inclusions coalesce so that 30 hr. after infection only one inclusion per cell remains. Six hours after infection the cells are as susceptible to further infection as uninfected cells.

INTRODUCTION

In conjunctival smears from patients with trachoma the proportion of cells that contain specific inclusions is usually small. It is therefore not surprising that cells containing more than one inclusion are rare; the number of infective organisms free at any one time would make multiple infection unlikely. Experimentally, cell cultures can be infected with a large number of trachoma organisms so that high multiplicities of infection can be achieved. Nevertheless, at the end of the growth cycle only one inclusion per cell is usually seen; it could arise from the union of several inclusions or from the development of the first organism to enter the cell, with the subsequent exclusion or inhibition of all others. The results of experiments done to distinguish between these possibilities are reported here.

METHODS

TRIC agents. The following strains were used; the suffix *f* denotes fast-killing variants (Taverne, Blyth & Reeve, 1964). The abbreviations used in this paper are given in parentheses.

TRIC/2/China/Peking-2/OT*f* (T'ang, Chang, Huang & Wang, 1957) (PK 2*f*).

TRIC/2/SAU/HAR-2/OT*f* (Murray *et al.* 1960) (SA 2*f*).

Preparation of pools. Pools were made from infected yolk sacs; they were treated with KCl and stored in 0·25 M sucrose in buffer as previously described (Taverne & Blyth, 1971).

Cell culture methods and media have been described (Taverne & Blyth, 1971).

Inclusion counts were made after centrifuging TRIC agents onto monolayer cultures of BHK 21 cells seeded 24 hr. before. Cultures were fixed with methyl alcohol and stained with Giemsa.

RESULTS

Cells infected with more than one organism and examined within 20 hr. of infection often contain more than one inclusion, although by 30 hr. there is rarely more than one inclusion per cell. Thus more than one organism must be able to enter a cell within the short period allowed for infection and, in these circumstances, at least the early stages of replication proceed normally. To investigate the apparent disappearance of inclusions, cell cultures were infected with 15 inclusion-forming units of strain PK 2*f* per cell and examined microscopically during the period 18–28 hr. after infection. Cells containing inclusions apparently in the process of coalescence were repeatedly observed (Plate 1). The time course of coalescence of inclusions was therefore determined by counting the inclusions per cell at various times (Table 1).

Inclusion counts could not be made with confidence earlier than 17 hr. after infection. At this time there was an average of six, indicating that some had already coalesced. From 17 to 24 hr. the number decreased progressively, until by 28 hr. no cells were observed with more than one inclusion. When many inclusions were grouped closely and were coalescing, it was sometimes difficult to decide whether they were still separated. Errors resulting from this difficulty would lead to an underestimate of the true number. After coalescence inclusions appeared larger and at a more advanced stage of development than those of equivalent age resulting from a single infection.

In the experiment just described all the infecting organisms must have been adsorbed by the cell within the 30 min. period of centrifugation. To determine whether the presence of a developing inclusion in a cell alters the chance of later infection, it was necessary first to define the time interval that would allow inclusions of different ages to be differentiated most clearly, and then to choose the time after infection most suitable for recognizing such inclusions. The initial bodies in inclusions become intermediate bodies and elementary bodies between 18 and 24 hr. after infection. Furthermore, during this time the diameter of inclusions resulting from infection with one organism almost doubled, so that inclusions of these ages could easily be distinguished.

To investigate the possibility that the presence of an inclusion changed the susceptibility of a cell to subsequent infection, cultures were first centrifuged with a concentration of organisms of strain SA 2*f* sufficient to infect most of the

Table 1. *The number of inclusions seen in BHK cells at various times after infection with* 15 *inclusion-forming units per cell of strain PK* 2*f*

Hour after infection	No. cells counted	Mean no. inclusions per infected cell	Range	± Standard error
17	67	6·1	2–22	0·38
18	49	4·4	2–11	0·32
20	71	3·2	1–9	0·23
24	60	1·8	1–7	0·16
28	> 100	1	1	—

cells, but such that there was less than a 1 % chance of multiple infection. Six hours later the cultures were again inoculated by centrifugation; they were fixed 24 hr. after the end of the first centrifugation, together with controls that had been infected only at one or other time (Table 2). Within each culture, counts were made in the same microscope fields, selected at random, of the cells that contained both 18 and 24 hr. inclusions (Plate 2), and of the number singly or multiply infected with inclusions of either age. The total number of cells in each field was also counted. Thus the chance of any cell becoming infected in either or both centrifugations could be calculated. The proportions of cells with 18 or 24 hr. inclusions in control cultures infected only at one or other time tallied with those in the doubly infected cultures.

Were the entry and development of new organisms not altered in infected cells, the chance of an infected cell supporting the multiplication of a second organism would be the same as that for any cell in the culture. Thus, in the first culture the chance of an inclusion developing within a cell after the first centrifugation was $\frac{64}{632}$. The chance of a second inclusion developing in a cell already bearing an inclusion was thus $\frac{64}{632} \times \frac{152}{632}$. In the 632 cells counted, the number of cells with an inclusion from both infections was predicted to be $\frac{64}{632} \times \frac{152}{632} \times 632 = 15$. This figure, and those predicted for the other three cultures, agrees well with the observed result, suggesting that our original assumption was correct.

DISCUSSION

The results reported here extend the observation made by Bernkopf, Mashiah & Becker (1962) and Kramer & Gordon (1971) that TRIC inclusions within a cell at first develop separately and eventually coalesce. Inclusions tend to aggregate in the area of cytoplasm near the nucleus, but contact is apparently not in itself sufficient to cause immediate coalescence since early inclusions frequently appeared to be touching each other but remained discrete.

A conclusion of practical importance is that in infectivity titrations based on inclusion counts in cell cultures spuriously low results can be obtained if multiple infection is not rigorously excluded.

As inclusions cannot be counted accurately earlier than 17 hr. after infection,

Table 2. *The number of cells containing inclusions in cultures inoculated at* 18 *and again at* 24 *hr with strain SA* 2*f*

	Cells with 18 hr. inclusions		Cells with 24 hr. inclusions		
No. of cells examined	Total	Cells with > 1 18 hr. inclusion	Total	Cells with 18 hr. inclusion in addition	Predicted no. cells with both 18 hr. and 24 hr. inclusions
632	152	21	64	18	15
592	88	12	78	10	12
335	114	33	60	19	20
299	51	6	48	9	9

the question whether coalescence occurred only after some hours of development or proceeded at a constant rate from the time of infection could not be answered directly. Statistical analysis of the numbers of inclusions present in infected cells at different times showed that the values fitted curves representing either possibility, and that the chances of one being a better fit than the other were not significantly different.

In the experiments with cultures infected twice, there was obviously a chance that within any cell an 18 and 24 hr. inclusion might coalesce. The degree to which such coalescence affected the results could not be calculated from the earlier experiment since there the multiplicity of infection was much higher, i.e. 15 organisms per cell as opposed to less than one. Nevertheless, the good agreement between the predicted and observed numbers of doubly infected cells suggests that such coalescence was not a significant factor.

The second infection of cells already bearing inclusions indicated that their surface was not altered in a way that excluded entry of another organism. Similarly, although TRIC organisms induce the formation of interferon and are susceptible to its action (Hanna, Merigan & Jawetz, 1967), any interferon present 6 hr. after infection did not prevent the apparently normal development of further inclusions.

The question has been raised as to how readily the conjunctiva of an individual already suffering from trachoma can be re-infected (Nichols, von Fritzinger & McComb, 1971). Our experiments, done in conditions where neither host defence mechanisms nor epidemiological factors operate, show that individual infected cells are completely susceptible to infection by organisms of the same strain at least for a few hours after entry of the first organism.

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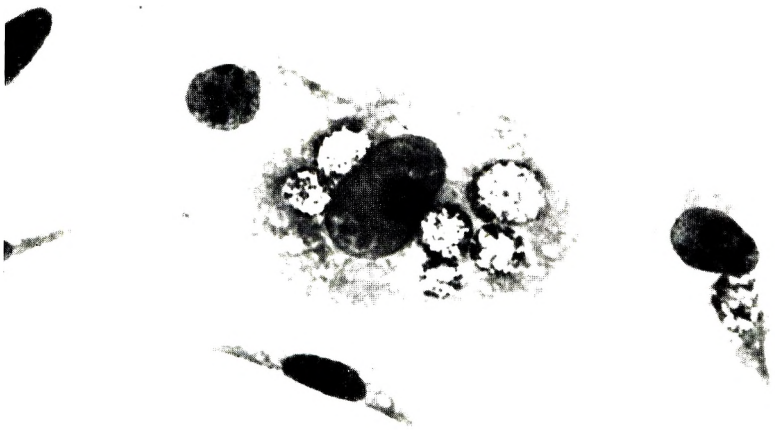

A

B

TAVERNE, J., BLYTH, W. A. & REEVE, P. (1964). Toxicity of the agents of trachoma and inclusion conjunctivitis. *Journal of General Microbiology* **37**, 271–5.

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EXPLANATION OF PLATES 1 AND 2

PLATE 1

BHK cells multiply infected with strain PK 2*f* 24 hr. earlier; (A) shows a cell with six discrete inclusions, (B) shows one with two inclusions that have apparently just coalesced.

PLATE 2

A BHK cell with two inclusions. The culture was infected twice with strain SA 2*f*, 24 and 18 hr. earlier. The inclusions resulting from each infection can readily be identified.

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The development of TRIC organisms in cell cultures during multiple infection

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SUMMARY

The relationship between multiplicity of infection and the yield of organisms and of polysaccharide was studied in BHK 21 cells infected with TRIC organisms. Although an increase in the multiplicity of infection resulted in an increase in the absolute number of organisms per culture, there was a decrease in relation to the number of infecting organisms. The amount of polysaccharide produced was independent of multiplicity of infection; it was not limited by the concentration of glucose in the medium. Polysaccharide leaked from cells and was slowly broken down in the culture medium.

INTRODUCTION

Although the agents of trachoma and inclusion conjunctivitis are usually isolated and grown in the chick embryo yolk sac, many workers have employed cell cultures (e.g. Bernkopf, Treu & Maythar, 1964; Gordon, Dressler & Quan, 1967; Jenkin, 1966). Cell cultures offer many advantages over the chick embryo yolk sac; infective organisms can be titrated with greater precision and they yield more easily purified material for biochemical investigations.

Within the genus *Chlamydia* an important species characteristic is the presence or absence in the inclusion of a carbohydrate which is probably glycogen (Gordon & Quan, 1965). The role of this material in the multiplication of the organism has not been elucidated, nor is it certain whether it is produced by the organism or the host cell (see Moulder, 1964).

Recently Blyth & Taverne (1972) observed that inclusions in multiply infected cells coalesce, and at a given time appear larger and more advanced than those derived from infection by a single organism. It seemed possible that in multiply infected cells developmental processes act synergistically. To improve our understanding of such processes, a quantitative study was made of the effect of multiplicity of infection on the number of elementary bodies produced, their infectivity and the yield of carbohydrate. This information is also of practical importance when large amounts of material are required, for example in biochemical studies or for production of vaccines.

MATERIALS AND METHODS

Strain

TRIC/ /China/Peking – 2/OT*f* (T'ang, Chang, Huang & Wang, 1957) was used; the strain is designated according to the system proposed by Gear, Gordon, Jones & Bell (1963) and the suffix *f* indicates a 'fast-killing' variant (Reeve & Taverne, 1964).

Preparation of pools

Suspensions of infected yolk sacs were treated with KCl and stored at $-70°$ C. (Taverne & Blyth, 1971).

Cell cultures

Monolayers of BHK 21 cells (Stoker & Macpherson, 1964) were seeded into Leighton tubes, 30 ml. or 250 ml. plastic bottles (Falcon Plastics) the day before infection. Growth medium was Eagle's tissue culture medium (BHK) (Wellcome Reagents Ltd) containing 0·035 % $NaHCO_3$, 10 % tryptose phosphate broth (Difco Laboratories) and 10 % calf serum (Flow Laboratories Ltd). The maintenance medium (MM) used after infection was growth medium without serum and contained streptomycin (100 μg./ml.). It was buffered to pH 7·5 with tris and HCl to a final concentration of 0·05 M tris.

Reagents

Phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954) without calcium and magnesium was used for washing cell monolayers.

Disodium ethylenediamine tetracetate (E.D.T.A.) 0·002 % in PBS was used to remove cells from the surface of bottles. Borate buffer pH 7·0 consisting of 0·02 M-H_3BO_3, 0·0008 M-$Na_2B_4O_7$, 0·0007 M-$CaCl_2$, 0·003 M-$MgCl_2$ and 0·15 M-NaCl in distilled water was used to resuspend cells after they were stripped from flasks.

Anthrone reagent was prepared by dissolving 0·5 g. anthrone (Hopkin and Williams Ltd) in a mixture of 500 ml. concentrated sulphuric acid (Analar) and 145 ml. distilled water (Mokrasch, 1954). It was stored at 4° C.

Assay of polysaccharide

Samples of infected and uninfected material were boiled with an equal volume of 10 N-KOH for 1 hr. to destroy any free glucose and then neutralized by glacial acetic acid (van der Vies, 1954). After suitable dilution, 1 ml. samples were heated with 6 ml. anthrone reagent at 80° C. for 40 min. and their optical density at 620 nm. was measured. The material measured in this procedure is referred to throughout as polysaccharide. Standard curves were made for each experiment with glucose (Analar B.D.H.) and oyster glycogen (B.D.H.). As little as 5 μg./ml. glucose could be detected with this assay method. Results are expressed in terms of glucose equivalents.

Infection of cell cultures and titration of infective organisms

Monolayers of BHK cells were infected with TRIC organisms in MM by centrifugation at 600 *g* for 30 min. at 35° C.; the medium was then replaced with new MM and the cultures were incubated at 35° C.

Inclusions were counted in cultures fixed with methyl alcohol and stained with Giemsa; the results were expressed as inclusion-forming units (IFU)/ml.

Particles were counted by a darkground method (Reeve & Taverne, 1962).

Design of experiments

Some uninfected BHK cultures were fixed and stained at the time of infection to determine the number of cells present. Other cultures were infected with various concentrations of TRIC organisms. The inoculum was also titrated so that the exact multiplicity of infection could be calculated retrospectively. After incubation for various periods some cultures were fixed to count the inclusions; in others the content of total particles, infective organisms and polysaccharide was assayed by the following methods.

Yield of new organisms. Culture vessels were incubated at 35° C. for 5 min. with E.D.T.A. and were then examined microscopically to ensure that all the cells had been removed from the surface. The cells were disrupted ultrasonically at 4° C. in a Soniprobe tank type 1130/2A (Dawes Instruments Ltd) at stage 8 for 30 sec. The disrupted material was then assayed for infectivity and for total particles.

Yield of polysaccharide. The MM from monolayers in 250 ml. flasks was discarded and the cells were stripped from the flasks into ice-cold borate buffer by rolling glass beads over the surface. The cell suspension was then disrupted with a mechanical shaker (Baird and Tatlock Ltd) at full speed for 2 min. If the polysaccharide was not assayed immediately the homogenized cell suspension was stored at −40° C.

RESULTS

Forty-five hours after inoculation inclusions resulting from infection with one organism differed strikingly in appearance from those derived from multiple infections (Plate 1).

Cultures that had been infected with < 1, 1·6, 8 or 16 organisms per cell were disrupted 28, 36, 42 and 48 hr. after infection and the numbers of infective organisms were titrated.

The yield of infective organisms from each inclusion increased with multiplicity of infection (Fig. 1). After 42 hr. inclusions resulting from infection with one organism contained on average 278 IFU whereas those receiving 16 infective organisms contained 848 IFU. Inoculation of more than 100 organisms per cell caused a cytopathic effect.

By contrast, increases in multiplicity of infection diminished the yield in relation to the number of infective organisms inoculated (Fig. 2). For instance, 42 hr. after infection, when all yields were attaining their maxima, the ratio of the yield

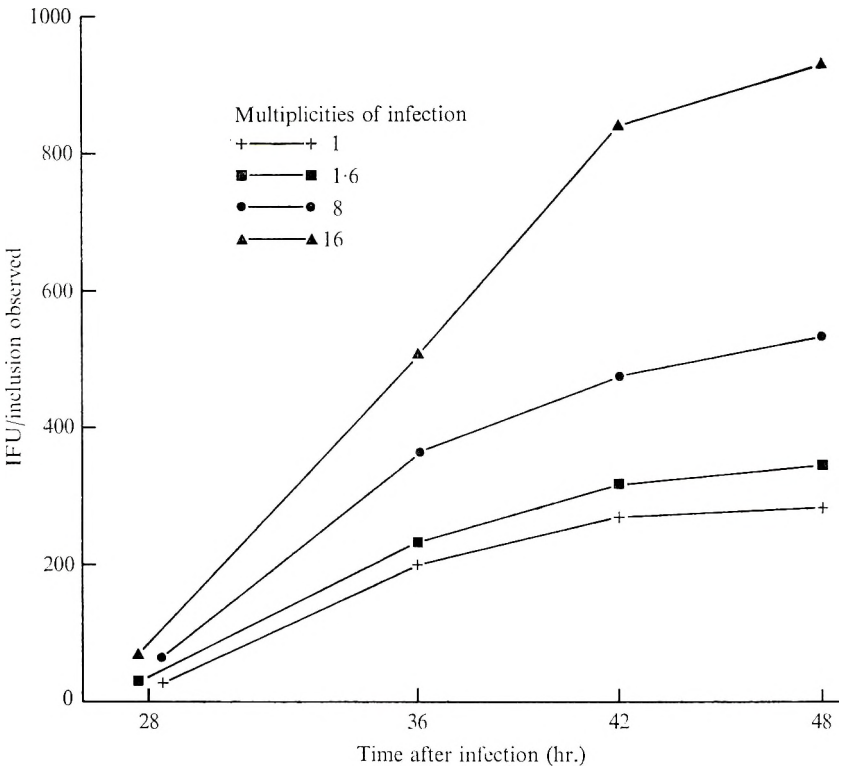

Fig. 1. The yield of infective organisms expressed as IFU/inclusion observed at each time.

Table 1. *Effect of multiplicity of infection on the yield of infective organisms and total particles per cell 42 hr. after infection*

Multiplicity of infection	IFU*	Mean	Total particles*	Mean
< 1	289 267	278	331 303	317
1·6	329 311	320	378 324	351
8	493 465	479	435 413	424
16	895 801	848	973 867	920

* Mean of two replicates.

of infective organisms to the number of infective organisms in the inoculum was 280 to 1 when the multiplicity of infection was 1 and 60 to 1 when the multiplicity was 16.

At all times, within the limits of experimental error, every new particle formed was infective. Table 1 gives the figures for 42 hr.

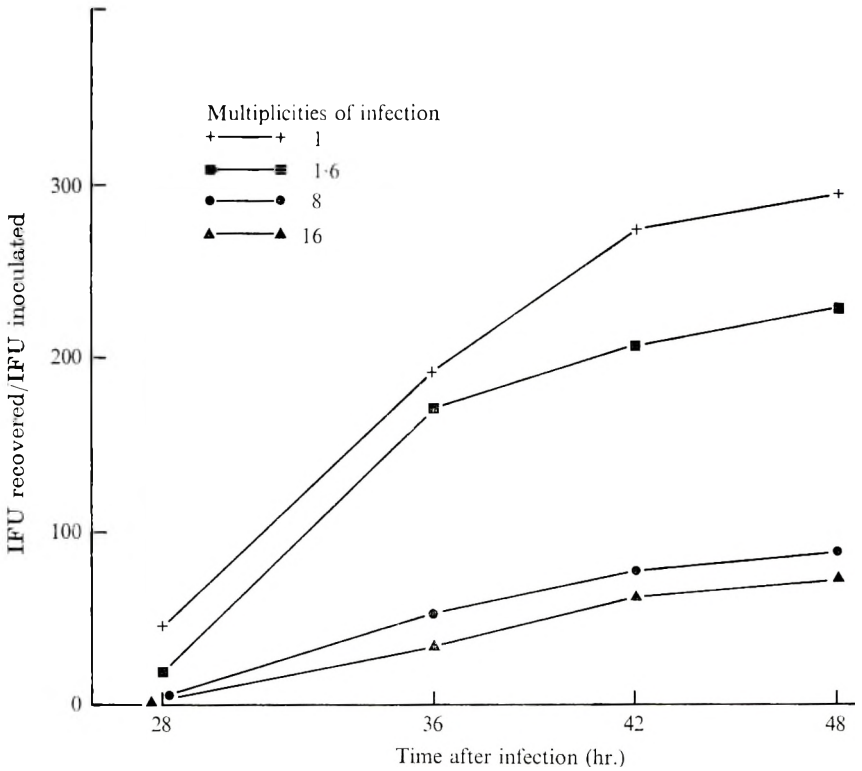

Fig. 2. The yield of infective organisms expressed as IFU recovered/IFU inoculated.

Yield of polysaccharide

Experiments were done to determine whether the amount of polysaccharide produced in infected cells varied with the multiplicity of infection in the same way as the number of organisms.

Polysaccharide was measured 29, 40, 48 and 56 hr. after infection of cultures with the same numbers of organisms per cell as before (Fig. 3). In uninfected cells the amount of polysaccharide did not vary with time. At no time did the amount per infected cell vary with multiplicity of infection. Polysaccharide was first detected 28 hr. after infection and the amount increased thereafter to reach a maximum, at 48 hr., of 115 μg. glucose equivalents per 10^6 infected cells. By 56 hr. the amount in the cells had decreased to 64·3 μg. glucose equivalents per 10^6 infected cells.

Effect of glucose concentration on the production of polysaccharide

The amount of polysaccharide formed by an infected cell at any time may have been constant, whatever the multiplicity of infection, because it was limited by the amount of glucose in the culture medium. The concentration of glucose in the medium of cultures infected with 8 organisms per cell was increased from 4·5 g./l. to 9 g./l., either immediately after inoculation, or 28 hr. later. The amount of polysaccharide formed was measured 42 hr. after infection.

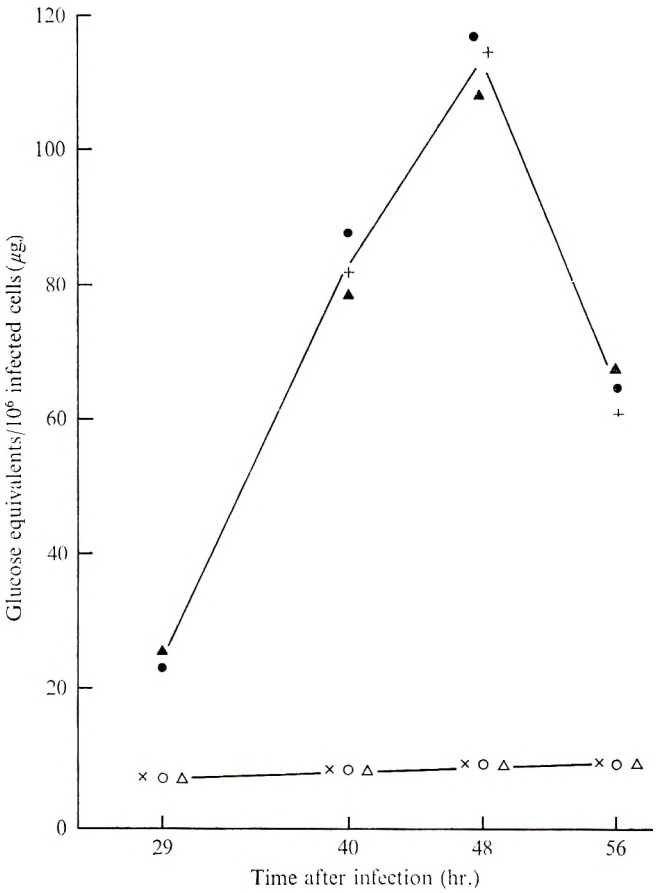

Fig. 3. Polysaccharide in infected and uninfected cultures. Key as in Fig. 1; closed and open symbols represent polysaccharide in infected and control uninfected cultures respectively.

The addition of more glucose to the medium did not affect the amount of polysaccharide formed per infected cell: there were 87·2 μg. glucose equivalents per 10^6 infected cells in the control cultures; 84·2 μg. when MM containing increased glucose was added immediately after infection and 88·2 μg. when it was added 28 hr. later.

Loss of polysaccharide from infected cells

The amount of polysaccharide in infected cells began to decrease 48 hr. after infection. It may have been degraded within the cell or may have leaked out of them. To test the second possibility the amount in the medium was measured at various times after infection.

Cultures were inoculated with eight organisms per cell and 30, 48 and 56 hr. after infection the MM was removed from these and similar control cultures and replaced with 5 ml. of fresh MM, the smallest volume that covered the monolayer. After incubation at 35° C. for 90 min. the polysaccharide in the new medium was assayed.

The amount and proportion of polysaccharide in the medium of uninfected cultures remained fairly constant. The amount in the medium of infected cultures was higher, but varied little with time; the proportion in the medium was least 48 hr. after infection, when the amount in the cells was greatest (Table 2).

Stability of polysaccharide in MM

The possibility that degradation of free polysaccharide in MM at 35° C. resulted in an underestimate of the true amount released by the infected cells was tested. A lysate was prepared from cells infected 48 hr. earlier and was heated at 100° C. for 20 min. to destroy enzymic activity; this treatment did not alter the amount of polysaccharide present.

Samples were then incubated at 35° C. for 6 hr. in the media listed (Table 3). Polysaccharide was assayed before and after incubation and a comparison was made with the stability of oyster glycogen in medium from infected cultures.

The amount of polysaccharide remained the same after incubation in either new or heated MM; in unheated media from infected and from uninfected cells the losses were respectively 32% and 15%. When oyster glycogen was incubated in medium from infected cultures a 42% loss was observed. The values obtained for the amount of polysaccharide in culture medium after 90 min. incubation are thus probably underestimated by the order of 5%, assuming that the rate of degradation is constant.

Table 2. *Polysaccharide in culture medium of infected and uninfected cultures*

	Uninfected μg. glucose equivalents/10^6 cells		Infected (8 IFU/cell) μg. glucose equivalents/10^6 cells	
Time	Monolayer	Medium	Monolayer	Medium
30	7·3	2·5	23·8	10·5
48	8·8	2·1	115·0	11·5
56	9·1	2·6	64·3	15·1

Table 3. *Stability of polysaccharide during 6 hr. incubation in MM*

Source of polysaccharide	Source of MM	Heated†	μg./ml. glucose equivalents of polysaccharide 0 hr.	6 hr.	% remaining at 6 hr.
Infected cell lysate*	New	No	82·5	85	103
	Infected culture	Yes	85	85	100
	Infected culture	No	82·5	56·2	68
	Uninfected culture	Yes	87·5	90	103
	Uninfected culture	No	85	72·5	85
Oyster glycogen	Infected culture	Yes	150	153	102
	Infected culture	No	150	87·5	58

* Cells infected 48 hr. earlier were disintegrated by shaking with glass beads; the lysate was heated at 100° C. for 20 min.

† 100° C. for 20 min.

DISCUSSION

Although many workers have studied the growth cycle of TRIC organisms only Furness & Fraser (1962) measured the yield of infective organisms from an inclusion. Using HeLa cells in conditions designed to ensure infection with only one organism, they obtained a maximum of 35–60 infective organisms per inclusion 34–38 hr. after infection. This figure is lower than the maximum of 278 reported here for BHK cells, but experience in this laboratory suggests that the difference is probably due to improvements in the method of titration.

These findings show that, within limits, increasing the multiplicity of infection resulted in an increase in the absolute number of organisms within each infected cell, and that these organisms were all infective on each occasion that they were tested, starting 28 hr. after inoculation. This finding is of practical value in devising conditions to give the greatest yield of infective organisms from cell cultures.

The observation that with multiple infection the yield of organisms is diminished in relation to the number of organisms inoculated, indicates that some kind of interference or competition occurs within the multiply infected cell.

The nature and function of the polysaccharide present in inclusions is not known. It stains brown with iodine and is frequently referred to as glycogen, but there is no evidence that glucose is the only sugar present or that the polysaccharide is not combined to another molecule. Regardless of the multiplicity of infection the same amount of polysaccharide was produced by each infected cell at any one time and was independent of the numbers of elementary bodies formed. This finding might indicate that the polysaccharide is a product of the host cell; but recent work by Jenkin & Fan (1971) on the preferential utilization of UDP-glucose by the glycogen synthetase of the mammalian host cell and of ADP glucose by Group A *Chlamydia* suggests that it is made by the organism.

Although Gill & Stewart (1970) found that the amount of glucose in the culture medium limited production of infective psittacosis organisms, their greatest concentration of glucose was 100-fold less than that used in xperiments reported here, in which yields of polysaccharide were not increased w n the concentration was doubled.

Bernkopf, Mashiah & Becker (1962) suggested that the olysaccharide might function as an energy reserve, 'used up during the final st es of maturation of the agent'. This hypothesis was based on the finding that the proportion of inclusions staining brown with iodine decreased between 28 and 48 hr. after infection, as the numbers of infective particles increased. By contrast, Reeve & Taverne (1967) found that the proportion of inclusions staining with iodine continued to increase after 28 hr. to reach a maximum at least 48 hr. after nfection. The ability of inclusions to be stained with iodine reflects to some extent their content of polysaccharide, but neither of these groups of workers measured this compound in inclusions.

Precise chemical measurements revealed a maximum 48 hr. after infection, about 20 hr. after first appearance of infectivity. There is no obvious explanation for the discrepancy between these results and those of Bernkopf *et al.* (1962), especially since in terms of infectivity the growth cycles were closely similar.

According to the suggestion made by Bernkopf and his colleagues, the decrease in amount of polysaccharide per inclusion during the later part of the growth cycle results from its biochemical degradation within the inclusion. However, the results reported here show that substantial amounts of polysaccharide appear in the medium of infected cultures. It is unlikely that this amount of free polysaccharide resulted from rupture of infected cells since it would be necessary to postulate that half the cells had lysed 30 hr. after infection to account for it, a notion not supported by microscopical examination of the cultures. The decrease in polysaccharide content of infected cells between 48 and 56 hr. after infection could be entirely accounted for by the observed rate of leakage of 10 μg. per hour.

I am grateful to Drs J. Taverne and W. A. Blyth for many valuable discussions, and to Professor L. H. Collier for his helpful criticism.

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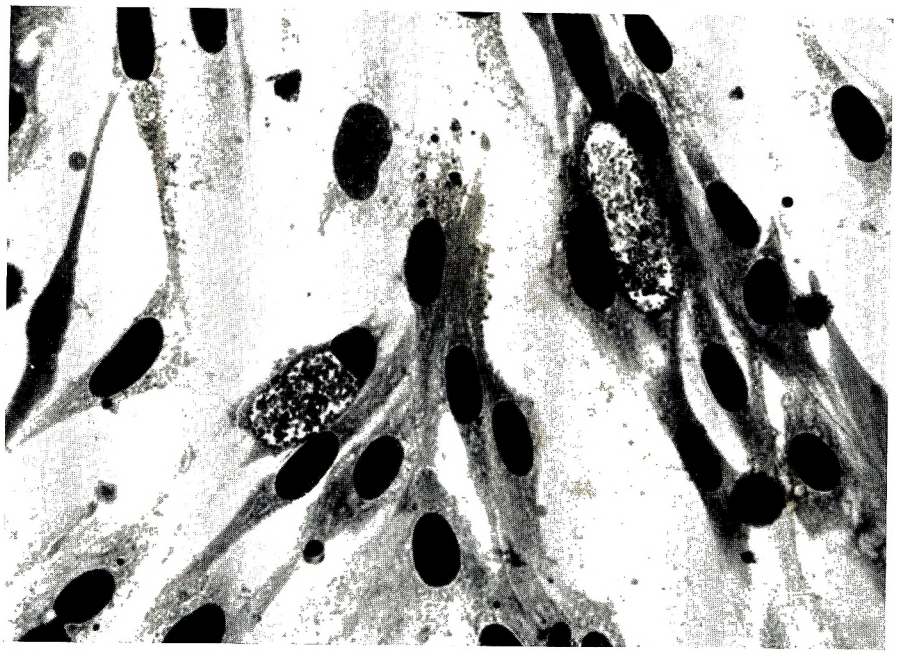
EXPLANATION OF PLATE

Fig. A. BHK-21 cells 45 hr. after infection with < 1 TRIC organism per cell. (× 480.)

Fig. B. BHK-21 cells 45 hr. after infection with 12 TRIC organisms per cell. (× 480.)



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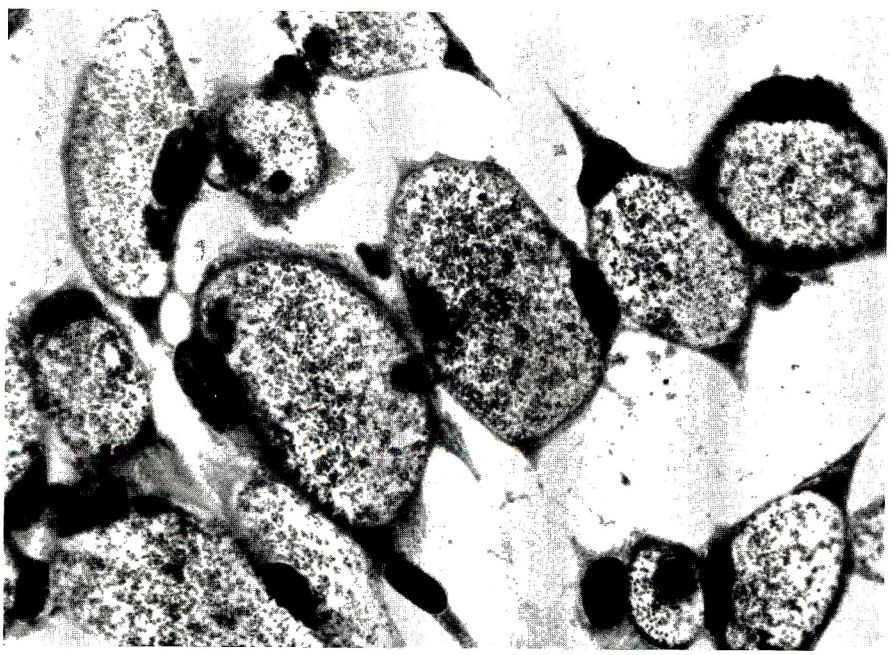

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An unusual plaque variant of rubella virus

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SUMMARY

In a comparative study of 24 different rubella virus strains, all but three formed small plaques in RK 13 cell cultures; of these one was the vaccine strain HPV-77, one was isolated from a congenitally infected infant, and the third was recovered from an adult who contracted severe rubella while handling this congenital strain. Whereas the plaque size of the vaccine strain was stable after further passage in cell culture, the plaque size of the other two rapidly diminished when the virus was passed in monkey kidney cells, and one of them was also reduced by passage in RK 13 cells. Cell culture passage of the typical small plaque strains did not result in altered plaque size.

INTRODUCTION

Lawrence & Gould (1969) showed that different rubella virus strains could be distinguished on the basis of plaque size and presented preliminary evidence that rapid changes in plaque size were associated with cell culture history. Evidence for this was also presented in a paper by Parkman, Meyer, Kirchstein & Hopps (1966), describing the vaccine strain HPV-77, the virus having changed from a small to a large plaque type during prolonged passage in monkey kidney cell culture; but it was not suggested that this change represented a profound alteration in the virus. It is generally accepted that rubella virus exists as one serotype (Best & Banatvala, 1970; Kono, 1969) although Fogel & Plotkin (1969) presented evidence which does not seem to support this view. The latter group used a plaque reduction technique for serotyping rather than the haemagglutination inhibition or tube neutralization methods employed by others. Whether or not only one serotype exists, it is clear that plaque morphology is not uniform, and this paper presents a comparative study of the plaque type of a wide selection of rubella virus strains and records in detail certain changes observed in two unusual cases.

MATERIALS AND METHODS

The twenty-four strains employed in this investigation are listed in Table 1 which shows the categories into which they fall and their cultural history. Virus was isolated from vaccinees or infected animals, from throat swabs, nasal swabs or washings, and from clinical material from the human fetus by macerating the sample in growth medium and subjecting it to three cycles of freezing and thawing

after which the fluid was clarified. The strains were subjected to passage in cell cultures using the simple procedure of harvesting inoculated cultures when extensive cytopathic effects developed or at 7–10 days after infection. All virus preparations were stored at $-70°$ C. or in the freeze-dried state at $-20°$ C.

The cell culture systems employed were RK 13 cells, WI-38 cells, Vero cells and primary cells from *Erythrocebus patas* monkey kidney, rabbit kidney or chick embryo. All cultures were incubated at 36·5° C.

The plaque test was carried out in 60 or 35 mm. Falcon plastic Petri dishes seeded with sufficient cells to form a confluent monolayer in 72 hr.; this was normally in the order of 200,000 cells/ml. The plates were incubated at 32·5° C. in an atmosphere of 5 % CO_2 in air, and were washed once with buffered Eagle's minimum essential medium (MEM) before use. The volume of inoculum was 0·2 ml. for 60 mm. plates, 0·1 ml. for 35 mm., and the period of virus adsorption was 15–30 min. at room temperature. The overlay medium consisted of Eagle's MEM, 0·09 % sodium bicarbonate, 2 % fetal or agamma calf serum and 1 % Difco Noble agar (10 ml. was required for 60 mm. plates, 4 ml. for 35 mm. plates). The plates

Table 1. *History and characteristics of rubella virus strains*

Strain	History	Plaque size (mm.)
Category I. Virus isolated from post-natal infection of children and adults. Throat and nasal swabs		
Day	RK 13 (3)*	0·75–1·0
Sheppard	RK 13 (6)	
Judith	YRK† (3)	
Lesley	P.D.‡	
Janine	P.D.	2·25–2·5
Category II. Virus isolated from congenital infection		
(*a*) Foetal liver and kidney		
He	RK 13 (1)	1·0–1·25
Wright	RK 13 (2)	
(*b*) Placental		
Savva	P.D.	1·0
(*c*) Post-natal throat swab, urine, lens		
Lambert	RK 13 (1)	0·75–1·0
Pullen (9 months)§	RK 13 (2)	
Andrews (6 days), Thomas E. (4 weeks)	RK 13 (3)	
Dunning (6 months)	RK 13 (4)	2·25–2·5
Thomas, T. (< 4 weeks)	RK 13 (7)	0·75–1·0
Simoni, Hitchcock	MK (2)	
Carnwright, Lefebvre (1), Gabriel, Goldthorpe	MK (3)	
Lefebvre (2)	MK (6)	
Category III. Vaccine strains		
HPV-77	MK (78)	2·0–2·25
Cendehill	YRK (51)	1·25–1·5
RA27/3	WI-38 (30)	1·0–1·25

* Number of passages.
† Primary rabbit kidney.
‡ Material plaqued direct from clinical sample.
§ Age of child when virus isolated.

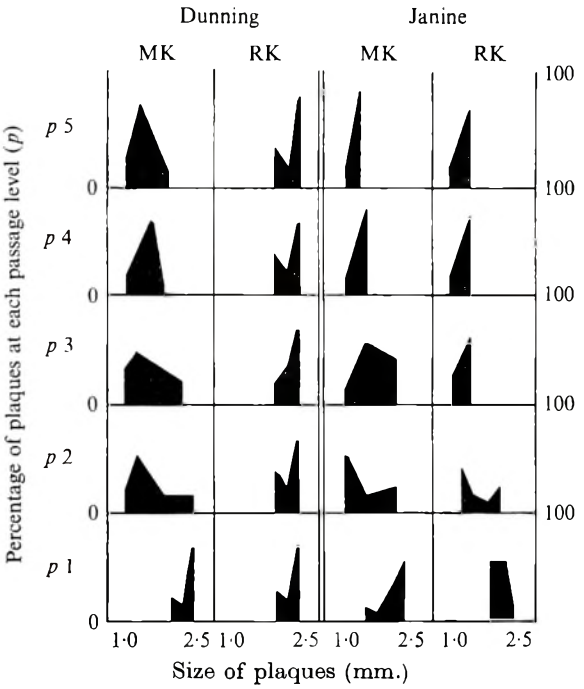

Fig. 1 Range of plaque size with passage.

were left at room temperature for 15–20 min. to allow the overlay to solidify before being returned to the cabinet. The cultures were incubated for 7 days, at which time the overlay was stripped off and the cell sheets stained with formalized crystal violet in phosphate buffered saline. Optimum results were obtained at 32·5° C. with RA27/3 virus and this temperature did not prejudice production of plaques by other strains. Deviation from this method resulted in either loss or variation in the size and quality of the plaques. Batches of agar were occasionally found to be toxic and changes in the concentration of agar or sodium bicarbonate adversely affected the test. Goat, rabbit, adult bovine, agamma and fetal calf sera were used experimentally for both growth and maintenance of the cells. Optimum results were obtained using adult bovine serum in the growth medium, and fetal or agamma calf serum in the maintenance medium.

RESULTS

Plaque sizes varied between 0·75 and 2·5 mm., the most common size being nearer the lower limit. Thus of the five strains examined in Category I, four formed plaques in the range 0·75–1·0 mm., and only one strain produced large plaques of 2·25–2·5 mm. Similarly, in category II most of the strains whether derived from fetal, placental or post-natal congenital material formed small plaques and only one strain produced a large plaque type. In the third category the vaccine viruses produced plaques in three size ranges, HPV-77 were large, RA27/3 were small and Cendehill formed plaques intermediate in size.

Selected virus strains were then passaged in continuous cell and in primary tissue cultures. On passage in RK13 and monkey kidney cells, only Dunning and Janine showed significant change in plaque size. Strain Janine in both cell culture systems reduced from large to small within six passages. Dunning only altered in this way when passaged in monkey kidney cells, six passages in RK13 leaving it unchanged (Fig. 1). Detailed studies of these changes indicated that a high percentage of the plaques were large in the original material and a high percentage were small after six passages. During the intermediate passage levels a range of plaque sizes were seen. We noted that the pattern of these changes was strikingly similar in all cases where they occurred. No significant changes in plaque size were observed in other virus strains subjected to passage in RK13 or monkey kidney, those tested being Sheppard and Lesley (Category I), Savva, Thomas and Dunning (Category II), HPV-77 and RA27/3 (Category III). Preliminary results indicate that Dunning is also reduced in size after passage in Vero cells but not in WI-38. Janine has not yet been tested in this manner.

HPV-77 passed in chick embryo or primary rabbit kidney showed no evidence of changes and similarly studies of RA27/3, HPV, Cendehill and Sheppard after passage through man or monkey indicated the stability of the plaque type.

DISCUSSION

Our studies show that limited passage of rubella virus in cell culture or animals does not normally result in the selection of different plaque types. Furthermore, it would appear that the usual plaque type is small whether obtained from pre- or post-natal infection, and even amongst the vaccine strains only the high passage virus HPV-77 produced large plaques. The large-type plaque, therefore, is unusual and the two low passage examples we describe may have some interest, although the rapid loss of this character discourages speculation about its genetic significance beyond the possibility that heterogeneity may exist. We note that one of the large plaque strains, Dunning, was obtained from a 6-month-old infant who had experienced congenital infection and the second, Janine, was from an adult case contracted whilst handling the Dunning strain. This infection was of some interest being relatively severe with widespread rash, enlarged glands, swollen joints and arthralgia.

It is tempting to suggest that the large plaque strain may have been selected during prolonged postnatal replication; however, the Pullen strain isolated from a 9-month-old child produced normal 1·0 mm. plaques as did the Andrews and Thomas strains from 4-week and 6-day-old infants respectively. Unfortunately we were not always able to obtain the information concerning the age of the infant when the virus was isolated – thus it is not possible to speculate further on the importance of the length of term of viral replication. Nevertheless, it is possible that the congenitally infected infant may be a potential hazard to the population due to the provision of an unusual ecological niche for the emergence of rubella virus variants.

We acknowledge the technical assistance of Terry Bromley and John Rowe.

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Host specificities of RNA phages

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SUMMARY

Host ranges of members of four groups of male-specific RNA coliphages were determined by plating on hosts carrying various derepressed plasmids. An RNA phage originally isolated on *Pseudomonas aeruginosa* failed to form plaques on any of the strains of *Escherichia coli*.

INTRODUCTION

The RNA phages of *Escherichia coli* adsorb to pili whose protein (pilin) is determined by a gene of the F (fertility) factor (Brinton, Gemski & Carnahan, 1964). The F factor and the col V factor (Kahn & Helinski, 1964; MacFarren & Clowes, 1967; Lawn & Meynell, 1970) are naturally occurring plasmids which synthesize pili constitutively. Many other plasmids, e.g. many of the antibiotic resistance (R) factors, carry genes specifying the formation of pili similar to those determined by the F factor (F-like pili) but the pilus genes are repressed and only a small proportion of host cells produce pili. Mutants constitutive for pilus production have been obtained from several of these plasmids (Meynell & Datta, 1967; Meynell, Meynell & Datta, 1968).

Although the pili produced by bacteria carrying derepressed F-like R factors are closely similar to those of bacteria carrying the F factor they are not identical. Lawn & Meynell (1970) showed that four classes of F-like pili could be distinguished serologically. For the purpose of this paper we have called the classes A, B, C and D. F and col V-K94 produced indistinguishable pili (class A). Pili determined by R 1 were unique (class B) as were those produced by R 538.1 (class C). Pili produced by cells carrying R 100, R 136 or R 192 constituted a fourth group (Class D).

Male-specific RNA phages fall into four groups distinguishable by a variety of physicochemical techniques (Watanabe, Miyake *et al.* 1967; Watanabe, Nishihara *et al.* 1967; Sakurai, Miyake, Shiba & Watanabe, 1968; Miyake, Shiba, Sakurai & Watanabe, 1969). The differences between these groups, in particular the antigenic differences, which imply differences in the conformation of the coat proteins, suggest that the adsorption specificities of the phages might not be identical. Silverman, Mobach & Valentine (1967) had previously shown that a small minority of the $MS2^R$ mutants of an Hfr strain of *E. coli* remained sensitive to phage $Q\beta$. We therefore decided to test the ability of representative phages from each of the four

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classes to form plaques on bacteria carrying plasmids specifying distinguishable F-like pili.

Two RNA phages infecting *Pseudomonas aeruginosa* have been reported (Feary, Fisher & Fisher, 1963; Bradley, 1966). These are very closely related (D. E. Bradley, personal communication). They adsorb upon pili produced by *Ps. aeruginosa* (Bradley, 1966).

MATERIALS AND METHODS

Phages

These are shown in Table 1.

Bacteria

J5.3 (*E. coli* K12 met F, proA, F^-) (Clowes & Hayes, 1968).

Plasmids

These are shown in Table 2.

Media

Nutrient broth, nutrient agar and top agar (Clowes & Hayes, 1968).

Table 1. *Phages used in experiments*

Phage	Group	References
male-specific		
MS2	I	Davis, Strauss & Sinsheimer (1961)
GA	II	Watanabe, Miyake *et al.* (1967); Sakurai, Watanabe & Ohno (1967)
QB	III	Watanabe, Miyake *et al.* (1967) Sakurai *et al.* (1967)
SP	IV	Sakurai *et al.* (1968) Miyake *et al.* (1969)
Pseudomonas		
Pp7		Bradley (1966)

Table 2. *Plasmids used in experiments*

Plasmids	Serological group	Resistances	References
F-like			
F	A	None	Cavalli, Lederberg & Lederberg (1953) Hayes (1953)
R1.19	B	kan*	Meynell & Datta (1967)
R538.1 drd	C	str, chl, sul	Cooke & Meynell (1969)
R192.7	D	str, tet, chl, sul	Cooke & Meynell (1969)
R136.8	D	tet	Cooke & Meynell (1969)
R384 drd	?D†	tet	S. Dennison, unpublished
R386 drd	?A?C†	tet	S. Dennison (1972)
I-like			
R64.11	—	str, tet	Cooke & Meynell (1969)
R144.3	—	kan	Cooke & Meynell (1969)

* A segregant of R1.19 carrying resistance to kanamycin only.

† Evidence presented in this paper.

Resistances: chl = chloramphenicol; kan = kanamycin; str = streptomycin; sul = sulphonamides; tet = tetracycline.

Phage adsorption

Host bacteria were grown overnight in broth, diluted tenfold in fresh broth and incubated without shaking for 2 hr. at 37° C. Unshaken cultures were used to favour pilus formation. Of this culture 0·25 ml. was added to 2·5 ml. of molten top agar at 46° C.; 0·2 ml. of the appropriate dilution of phage suspension was added and, after 10 min. for adsorption, the contents of the tube were poured onto a nutrient agar plate. Plates were incubated overnight at 37° C.

RESULTS

Efficiency of plating

Suspensions of the four phages were diluted to give approximately equal titres (10^9 plaque forming units per ml.) on J5.3 (F). Relative titres of these suspensions on various hosts are recorded in Table 3.

Three host range mutants of phage SP (SPh_1, h_2 and h_3) isolated from plaques on J5.3 (R1.19) are included in Table 3. These show a new host range.

Plaque morphology

On J5.3 (F), phage GA produced large plaques with well-marked fuzzy halos. Within the halos, 'satellite' plaques were seen (Pl. 1, fig. 1). On J5.3 (R538.1 drd) GA formed plaques with less well developed 'satellite' systems (Pl. 1, fig. 2). Plaques with well-marked, rather fuzzy halos seem to be the norm for all the phages tested, but on J5.3 (R538.1 drd) phage SP formed plaques with sharp edges (Pl. 2, fig. 3). On J5.3 (R386 drd) all four phages plated with high efficiency but produced very turbid plaques, rather difficult to count (Pl. 2, fig. 4).

On J5.3 (R1.19) phage Qβ produced plaques with low efficiency. The plaques were very variable in size and, probably, many were too small and faint to be counted. Such a result is predictable when the receptors are poorly adapted for the phage.

Phage Pp7 did not form plaques upon any of the tested strains of *E. coli* carrying derepressed R factors of F or I type.

Table 3. *Relative efficiencies of plating of phages on various hosts*

	Host							
Phage	J5.3	J5.3 (F)	J5.3 (R1.19)	J5.3 (R538.1 drd)	J5.3 (R192.7)	J5.3 (R136.8)	J5.3 (R384 drd)	J5.3 (R386 drd)
MS2	0	1	$1{\cdot}5 \times 10^{-1}$	$1{\cdot}9 \times 10^{-1}$	$3{\cdot}5 \times 10^{-4}$	$2{\cdot}5 \times 10^{-4}$	$1{\cdot}2 \times 10^{-4}$	8×10^{-1}
GA	0	1	0	2·9	0	0	0	2×10^{-1}
Qβ	0	1	$2{\cdot}5 \times 10^{-5}$	2·0	$5{\cdot}6 \times 10^{-5}$	$4{\cdot}0 \times 10^{-5}$	$4{\cdot}3 \times 10^{-5}$	3×10^{-1}
SP	0	1	2×10^{-7}	0·9	0	0	0	10^{-1}
SPh_1	0	1	1	1·9	n.t.	0	n.t.	10^{-1}
SPh_2	0	1	1	1·2	n.t.	0	n.t.	4×10^{-1}
SPh_3	0	1	8×10^{-2}	5×10^{-1}	n.t.	0	n.t.	2×10^{-1}

n.t. = Not tested.

DISCUSSION

All RNA phages so far described adsorb to pili. The genetic determinants responsible for the receptor pili of *Pseudomonas aeruginosa* (Bradley, 1966) and *Caulobacter* sp. (Schmidt, 1966) are unknown, but all the RNA phages of *Escherichia coli* adsorb to F-like pili.

In nature, F-pili are produced either constitutively as with F or col V factors, or by cells carrying plasmids whose pilin-producing genes have escaped repression by some physiological peculiarity, e.g. by cells which have recently accepted the plasmid (Watanabe, 1963). So far as is known, the only naturally occurring plasmids which produce F-pili constitutively form pili of serological class A. All the phages tested adsorb efficiently to pili of this type. This may be because all isolations of male-specific phages have been made using bacteria carrying the F factor. Alternatively the selective advantage of being able to adsorb to constitutively produced pili may be so great that no phages have been able to evolve exclusively an adsorption specificity for pili whose synthesis is normally repressed. The point could be settled by making a survey of male specific phages isolated by adsorption to pili distinct from those determined by F (such as those specified by R1, R136 or R192).

Pili produced by J5.3 (R386 drd) seem to have a specificity of adsorption similar to those of J5.3 (F) and J5.3 (R538.1). Compatibility studies and inhibition of plaque formation by phage T7 on R386 suggest that this plasmid resembles F more closely than do other F-like R factors (Dennison, 1972).

The adsorption specificities of the various RNA phages may throw light on the selective pressures that have led to the evolutionary diversification of sex pilus specificity. Most of the R factors tested produce pili less efficient as phage receptors than those encoded by F (R538 is the only exception). The widespread prevalence of RNA phages capable of adsorbing to sex pili must favour mutant plasmids, coding for pili that are not efficient phage receptors (though still efficient for plasmid transfer).

Phage adsorption specificities of the various pili may be compared with the serological relationships (Lawn & Meynell, 1970). Serologically R136 and R192 pili are indistinguishable and it is satisfying to find that their phage adsorption properties are closely similar. R384 determines very similar pili judging by phage adsorption specificity.

R538.1 pili are serologically intermediate between those encoded by F (or col V-K94) and those of R1. Thus, anti R538.1 pilus serum adsorbed with R1 pili, still reacted with F (or col V-K94) pili and serum adsorbed with F (or col V-K94) pili could still react with R1 pili (Lawn & Meynell, 1970). Phage specificities of F and R538.1 pili are very similar, confirming the serological relationship. On the other hand R538.1 pili and R1 pili have very different phage receptor properties. Perhaps the R1 pilus specificity (class B) arose as the result of a mutation to resistance towards phages of groups II and IV in a plasmid previously determining a pilus similar to that of R538.1. This hypothesis is open to experimental test.

Serological comparisons of RNA phages have lead Miyake *et al.* (1969) to postulate that groups I (e.g. MS2) and II (e.g. GA) are related and that groups III (e.g.

Qβ) and IV (e.g. SP) are also related. However, phages GA and SP show similar host ranges differing from those of MS2 and Qβ. (This is especially marked on hosts producing pili of class D.) Thus the host range patterns do not support the proposed relationships.

Plaque formation by phage Qβ on J5.3 (R1.19) is a very inefficient process. Only a small proportion of the phage particles form countable plaques. These plaques are heterogeneous in size and when the plates are inspected carefully one gets the impression that there are numerous plaques too small or too turbid to be counted. This is what might be expected for plaque formation by a phage poorly adapted for adsorption to the receptors on a host. Probably among the phage particles from the largest plaques one would find host range mutants better adapted to adsorption on the host. We looked at phage from plaques formed by phage SP on J5.3 (R1.19). The efficiency of plating was less than 10^{-6}, but the plaques formed were clear, suggesting that the phages adsorb efficiently. As might be predicted, they resulted from host range mutants with new specificity patterns (Table 3).

Very little is known about the details of the interaction between RNA phages and pili. It is not even possible to predict whether phage mutations altering the host range should specify alterations in the major coat protein (Weber & Konigsberg, 1967) or a minor component (Roberts & Steitz, 1967). This question can be settled using these mutants. Since it is possible to obtain both pili (Brinton, 1965) and phages (Gesteland & Boedtker, 1964) in large quantities such mutants and mutant sex pili unable to adsorb particular phages may be useful in investigations of the specificity of interaction between the protein molecules of phage and receptor.

The peculiar 'satellite-ringed' plaques of phage GA may be produced by a mechanism similar to that responsible for star mutant plaques of phage T2 (Symonds, 1958), and comparable plaque mutants of several other phages (reviewed by Symonds, 1958). If phage GA is inhibited from lysing infected cells near the edge of the plaque (possibly because of superinfection) the fuzzy edges of the plaques would be explained. Mutants (not susceptible to the inhibition) arising in the peripheral regions of the plaque would be expected to form satellite plaques of the type observed.

The pseudomonas phage Pp7 failed to form plaques on any of the strains of *E. coli* tested. It seems that it must adsorb to pili of a specificity different from either F or I.

We are very grateful to Dr T. Miyake, Dr D. E. Bradley and Dr I. Watanabe for kindly supplying the RNA phages. We thank Dr N. Datta for supplying the plasmid-containing bacterial strains and for many helpful discussions.

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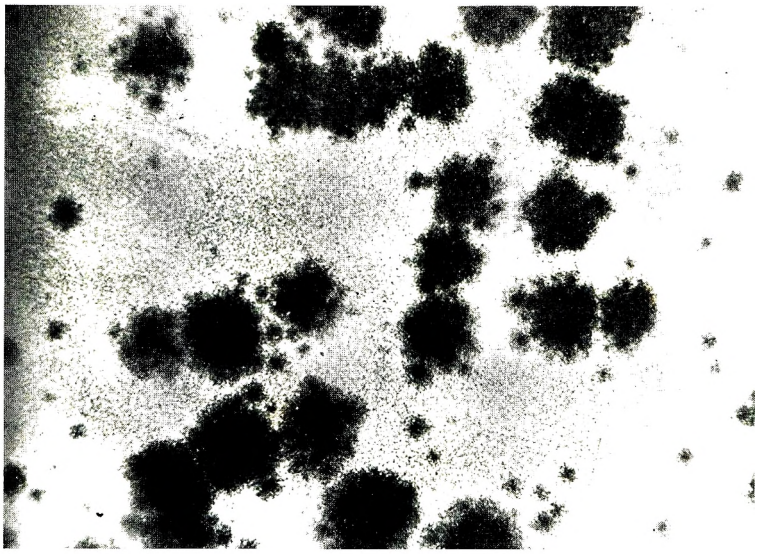

Fig. 1.

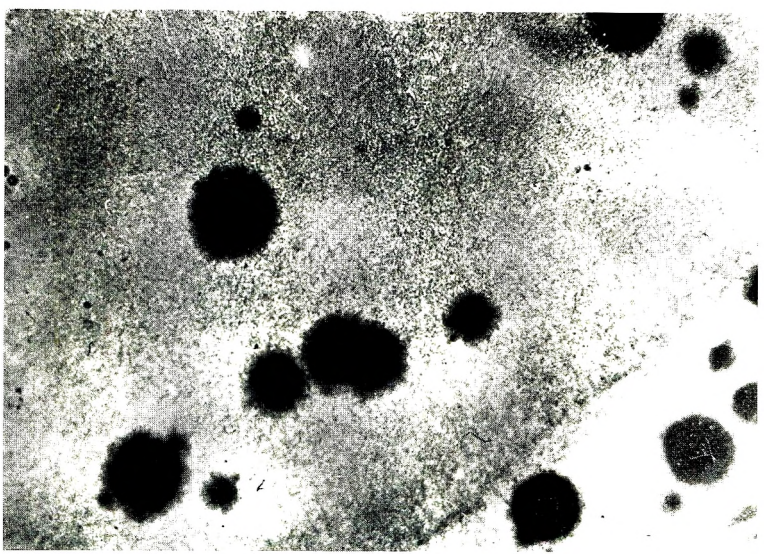

Fig. 2.

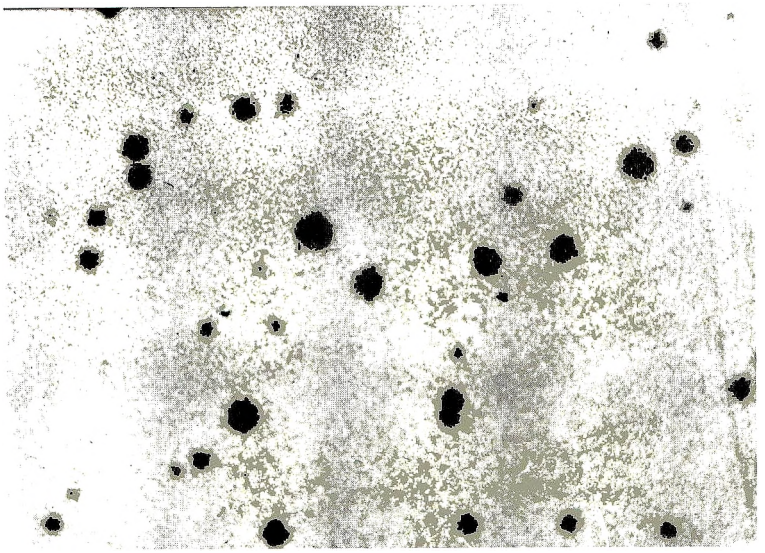

Fig. 3.

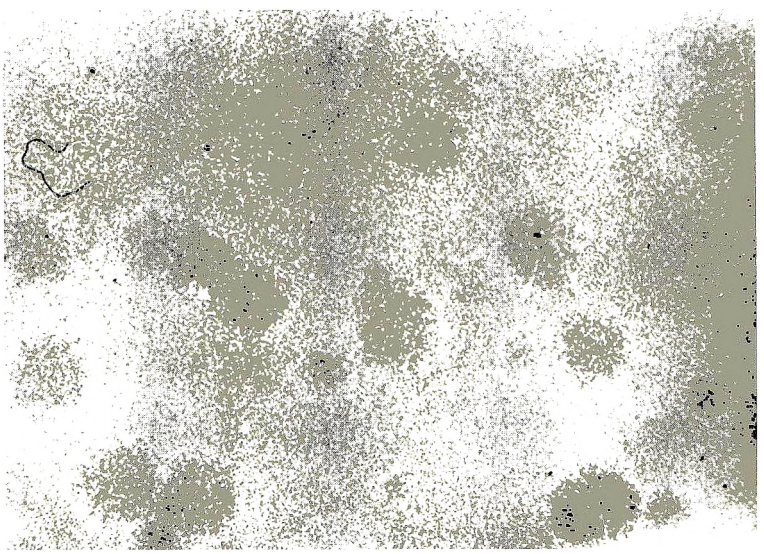

Fig. 4.

EXPLANATION OF PLATES

Plate 1

Fig. 1. Plaques formed by phage GA on J5.3 (F).

Fig. 2. Plaques formed by phage GA on J5.3 (R538.1 drd).

Plate 2

Fig. 3. Plaques formed by phage SP on J5.3 (R538.1 drd).

Fig. 4. Plaques formed by phage GA on J5.3 (R386 drd).

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With 1 *plate*
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Contamination of an operating theatre by Gram-negative bacteria. Examination of water supplies, cleaning methods and wound infections

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SUMMARY

This paper describes a search for Gram-negative bacteria in an operating theatre and the steps taken to reduce the level of environmental contamination.

A high rate of infection in clean wounds prompted a bacteriological survey. Potential sources of infection found, and the measures employed are described in the hope that others may be encouraged to examine familiar equipment critically and to improve hygiene even in old premises.

The choice, design, use and care of cleaning and sterilizing equipment were open to criticism. In particular, a currently popular floor-scrubbing machine provided a breeding ground for *Pseudomonas aeruginosa* and was distributing it in the theatre environment.

INTRODUCTION

During the first 3 months of 1970 the rate of Gram-negative infection of clean wounds increased from 10% to 18% (Table 1) among patients operated upon in a single theatre which was used for both clean and dirty cases. Johnstone's (1970) definition of a dirty operation as one performed in the presence of sepsis, or procedures such as anal operations and colostomy, is followed.

The information concerning infected wounds was gathered from data accompanying pus and swabs sent to the laboratory at the discretion of the clinicians concerned.

A preliminary inspection of the operating suite in April led to a continuous bacteriological survey during which a series of repairs, improvements and hygienic measures was implemented to reduce the level of bacterial contamination of the environment.

METHODS OF THE SURVEY

Nose and throat swabs were taken from the theatre staff. An initial sanitary inspection of the theatre premises was followed by weekly visits and interviews with the staff. Twenty-one sites were cultured, many of them at weekly intervals (Fig. 1). Cotton-wool swabs, moistened on the medium for dry sites, were rubbed over an area of about 10 cm.^{2} and then on blood agar and MacConkey agar plates incubated overnight at 37° C. Bacteria were identified by conventional methods. A sample of the pseudomonas strains isolated was typed by the Cross-Infection Reference Laboratory at Colindale.

RESULTS OF THE SURVEY

Nose and throat swabs from the theatre staff revealed neither Gram-negative infection nor penicillin-resistant staphylococci.

The theatre suite consisted of a main operating room and six other rooms interconnected and opening to one corridor. The unit was old and some repairs asked for had not been carried out. There was no air conditioning, the floor was cracked and a door and window broken. At the first survey in April, 13 sites in the theatre which were cultured all yielded confluent growth of *Pseudomonas aeruginosa* and other Gram-negative organisms.

Fig. 1. shows diagrammatically the results of cultures from various sites, with some notes on measures taken. Each square on the figure shows the result at one site. Only a sample of the total pseudomonas cultures was typed. Six of the 11 environmental strains typed were of serotype 10, phage pattern (100 RTD) 44/68+ and such strains are shown as 'endemic' in the figure.

Table 1. *Clean procedures in an operating theatre during* 10 *months with the organisms isolated from subsequent wound infections*

	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.
Total procedures	52	50	40	50	36	35	30	20	30	41
Wound swabs sent to laboratory	15	17	10	16	14	19	8	7	10	10
No bacteria found on culture	4	1	1	4	2	5	4	2	4	2
Gram-positive bacteria										
Penicillin resistant *Staphylococcus aureus*	4	7	3	2	1	3	1	3	0	2
Penicillin sensitive *Staphylococcus aureus*	0	1	0	2	3	3	0	1	3	3
Other Gram-positives streptococci and skin commensals	2	2	1	3	3	5	1	0	1	1
Total Gram-positives and percentage clean procedures	6 (12%)	10 (20%)	4 (10%)	7 (14%)	7 (19%)	11 (31%)	2 (7%)	4 (20%)	4 (13%)	6 (15%)
Gram-negative bacteria										
Pseudomonas aeruginosa	0	1*	2*†‡	3*‡‡	0	0	1	0	0	0
Klebsiella species	0	1	0	2	1*	1	0	1	0	0
Common gut species, *Escherichia coli*, *Proteus* species, etc.	5	5	5	1	5	4**	1	0	2	3
Total Gram-negatives and percentage clean procedures	5 (10%)	7 (14%)	7 (18%)	6 (12%)	6 (17%)	5 (14%)	2 (7%)	1 (5%)	2 (7%)	2 (5%)

* Staphylococcus also present in one swab.
† Haemolytic streptococcus also present in one swab.
‡ Endemic strain of *Pseudomonas aeruginosa* also present in one swab.

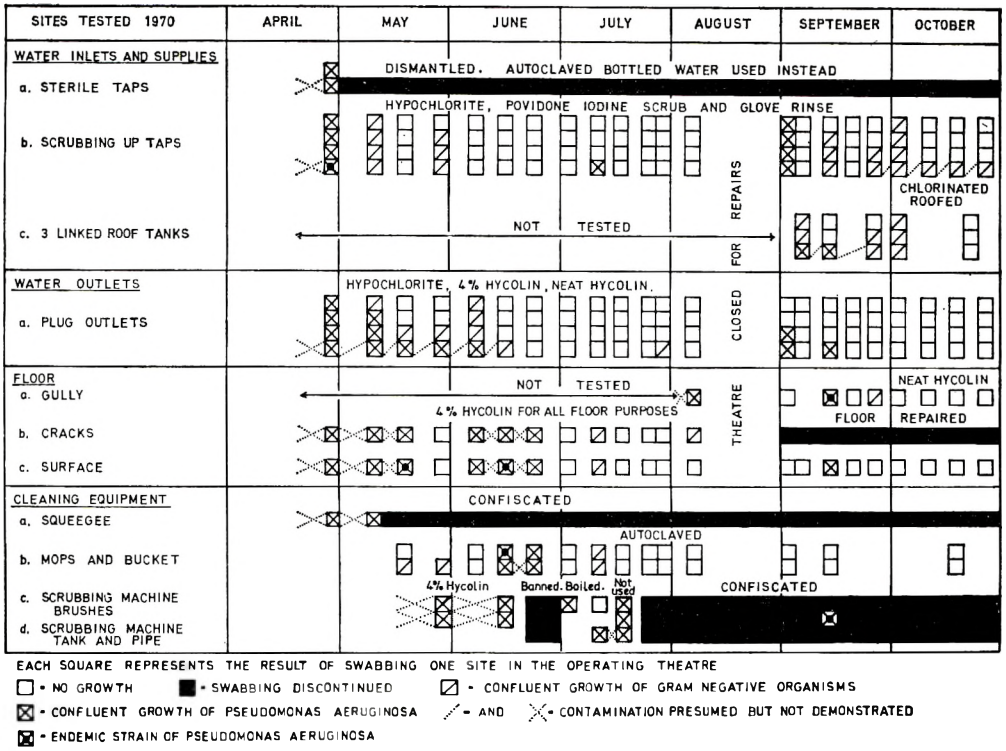

Fig. 1. Sites in the operating theatre contaminated by *Pseudomonas aeruginosa* and other gram-negative organisms.

Five main potential sources of infection were discovered.

1. Plumbing supplies and outlets.
2. The floor.
3. Floor-cleaning methods.
4. Instrument sterilizing methods.
5. Maintenance of premises.

Plumbing

Sterile water

Fixed in the wall of the main theatre were taps from a 'sterile' water supply used for moistening swabs and rinsing instruments. Profuse Gram-negative bacilli were isolated from these taps. The 'sterile' water apparatus was removed and bottled autoclaved water supplied by the dispensary instead.

Washing

Eight washing sinks in the suite had mixer taps for cold and hot water, which was often tepid. Cold water was drawn from linked roof tanks which were not inspected until September when they were found to be bird-fouled, without covers and containing two dead pigeons. The tanks were cleaned out and superchlorinated and the temperature of the hot water supply was increased. Profuse pseudomonas

and other Gram-negative bacteria were isolated from all the taps. Spray roses were removed and a syringe and catheter was used to inject 40 ml. of a 2 % solution of hypochlorite (20,000 parts per million available chlorine) into each tap each evening. This treatment produced negative cultures in general, but contamination recurred occasionally. Broken contaminated hexachlorophane dispensers were replaced by a povidone iodine dispenser beside each hand-washing sink.

Water outlets

Stagnant water lay in U-bend waste traps below each plughole and four sinks also had inbuilt overflow traps. An outflow trap leading from a gully in the floor of the main theatre was unclean. Swabs taken from plugholes and U traps produced, as had been expected, a confluent growth of Gram-negative bacilli. An attempt was therefore made to find whether contamination might have spread upward from plugholes. Blotting paper cards were fixed over several taps so that the faucet protruded. Methylene blue was put in and around the plughole and the tap turned on. Blue splashes appeared on the blotting paper showing that water from the plughole area could be splashed up inside the faucet.

Another test was made with blotting paper attached above waist level at the front of a gown. Blue splashes appeared at distances up to 18 in. above the plughole and across a width of 17 in. More sophisticated splash tests have been made by Kohn (1967). Following this evidence, sink plugs were discarded and plugholes and overflow pipes were treated with 40 ml. neat Hycolin each evening, which eliminated bacterial growth. Previously 2 % hypochlorite and then 4 % Hycolin solutions had been ineffective. The four sinks with overflow fitments are to be replaced.

Floor

Although repairs had often been requested, a cracked terrazzo floor had broken vulcanite fillets and an attempt had been made to bridge cracks with sticking plaster to prevent accidents. The surface, the cracks, and the plaster were heavily contaminated with *Ps. aeruginosa* of the endemic type (Fig. 1). This was traced to cleaning equipment (see below).

The floor was re-surfaced.

Footwear

Sterile bootees were placed outside the theatre suite, to slip over outdoor footwear. A bin was provided for those discarded after leaving the theatre. This simple arrangement needed careful supervision to prevent the mixing of clean and dirty bootees.

Floor-cleaning methods

Cleaning of the operating suite was the responsibility of a theatre sister, not the domestic superintendent. It was carried out by a domestic worker and theatre porters.

Mops

The disinfectant policy of the hospital recommended mops with heads which could be autoclaved, but unsterilizable string mops and squeegees were still being used in the theatre. A string mop was used with detergent each morning and also for mopping up spilt blood. After use it was returned to a bucket of Hycolin. No one was prepared to name the age or concentration of this disinfectant, which yielded a confluent growth of the endemic pseudomonas (Fig. 1).

Mops with detachable heads were introduced. These were supposed to be autoclaved daily, but persistent contamination made it clear that, initially, this was not being done. Considerable effort was required to ensure that the mop heads were actually autoclaved. Bacteria then ceased to live on the mops.

Scrubbing machines

The floor was scrubbed daily by a modern machine and then partially dried with a squeegee and mop. A second modern machine of another make had stood unused for some time. It had been tried once, when the tank was found to be too heavy for one person to empty.

The scrubbing machine in use was examined in detail. Three revolving brushes were supplied with water through a long narrow bent junctioned pipe, leading through a plastic valve from a fibreglass tank with a smooth outer surface and rough inner surface. The brushes were washable and could be autoclaved, but the feedpipe and tank could not.

Before the survey, Teepol detergent had been used in the machine. This was replaced by 2% Hycolin and then because floor contamination persisted, by 4% Hycolin, which is twice the recommended concentration. The machine brushes, nevertheless, showed very heavy contamination with *Ps. aeruginosa* and other bacteria, so they were autoclaved, but after return to use they were found to be as heavily contaminated as before. Only then was the fluid inside the tank, nominally 4% Hycolin, cultured. More than 10^5 bacteria per ml., mainly pseudomonas, were counted (Plate 1). Since it was not feasible to sterilize the tank or feedpipe, the use of the machine in the operating theatre was stopped. Autoclavable mops and scrubbing brushes, each detachable from its long handle, were obtained and the floor was cleaned with 4% Hycolin.

The scrubbing machine was studied further in the laboratory. The tank and the feedpipe leading to the brushes contained a sludge of micro-organisms, predominantly *Ps. aeruginosa*, and the endemic strain was still present when the machine had been out of use for 7 weeks (Fig. 1). Experiments showed that a spray of dirty water could be collected at a distance of 2 ft. from the machine, both at floor level and 1 ft. up the wall. Gram-negative organisms were grown on plates exposed in both these positions. Fig. 2 shows the direction of the spray thrown out by the machine. A sink at a distance greater than 6 ft. from the machine might receive organisms in the spray, depending on droplet size and incident draughts.

With the help of Mr R. Barfield of the Maintenance Department a machine provided by the manufacturers for experimental purposes has been reconstructed.

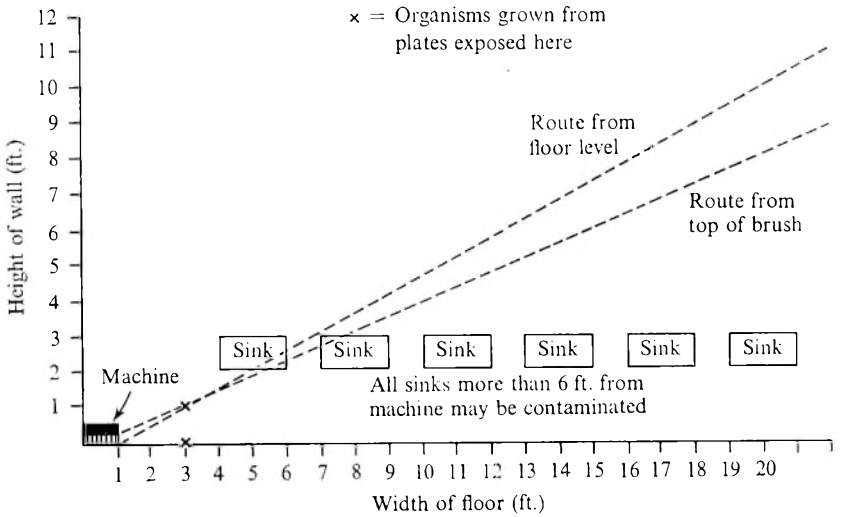

Fig. 2. Possible route of micro-organisms from scrubbing machine to sinks.

The modification has a stainless-steel tank and valve connected to a short length of transparent tubing. These are easily removed as a unit for washing and autoclaving. A rubber mudguard around the floor unit reduces the height of splash from the rotating brushes to under 3 in.

Instrument sterilizing methods

Although inspected regularly by the makers, an obsolescent autoclave in the operating suite was not working efficiently. Centralized steam pressure varied but it has now been controlled by a new regulator. It has been agreed that every autoclave in use shall be tested daily and maintained as described in *Hospital Technical Memorandum* No. 10 (1968). A central sterile supply department service for porous loads is being arranged.

A boiling water 'sterilizer' was in use for bowls and some instruments, producing steam which condensed on surfaces. The use of this apparatus for instruments in the theatre was discontinued as recommended by the Department of Health (1969). In any case it was found to be uninsurable.

Maintenance of premises

In addition to the dilapidated floor and water tanks described above, a broken door and window permitted draughts from a pigeon-fouled area. Discarded dressings and wrappings were in dustbins obstructing a fire escape. Ill-fitting lids allowed the contents to scatter as litter.

The floor, door and window were repaired and the tanks cleaned and roofed. The fire escape was cleared and arrangements made for theatre refuse to be collected in plastic bags and taken directly to the incinerator.

Table 2. *Number of operating theatre sites found contaminated by Gram-negative bacteria*

Number of sites	Apr.	May	June	July	Aug.	Sept.	Oct.
Swabbed	13	36	38	66	13	63	48
Contaminated by Gram-negative organisms	13 (100)	24 (67)	17 (45)	11 (17)	2 (15)	22 (35)	10 (21)
Contaminated by *Pseudomonas aeruginosa*	13 (100)	12 (33)	13 (34)	6 (9)	1 (8)	12 (19)	0 —

The figures in parentheses are percentages.

Table 3. *Wound swabs examined before and after implementation of hygienic measures in operating theatre environment*

	Jan. to June	July to Oct.
Clean incisional operations	263	121
Wound swabs sent to laboratory	91	35
Gram-positive organisms found	45 (17)	16 (13)
Staphylococcus aureus found	29 (11)	13 (11)
Gram-negative organisms found	36 (14)	7 (6)
Pseudomonas aeruginosa found	6 (2)	1 (1)

Figures in parentheses are percentages of the total operations in each group.

RESULTS OF HYGIENIC MEASURES

Details of the growth of Gram-negative organisms from a variety of sites in the operating theatre are shown diagrammatically in Fig. 1 and summarized in Table 2. Apart from the repair of the theatre floor, and the treatment of the water tanks on the roof, most improvements in hygiene were introduced before or during July. Perhaps some of the increased contamination in September came from the pigeon-fouled water supply and as a result of floor repairs. Table 3 shows the numbers of operations and of wound swabs sent to the laboratory before and after the introduction of these measures, and is a summary of the detailed information set out in Table 1. During the period from January to October, inclusive, 384 clean procedures were undertaken in this theatre and 43 Gram-negative wound infections including seven with pseudomonas present were diagnosed in the laboratory (Tables 1 and 3).

After June the percentage of swabs yielding Gram-negative organisms was halved (from 14 % to 6 %) as was the proportion yielding *Ps. aeruginosa* (from 2 % to 1 %) (Table 3). The temporal correlation of Gram-negative organisms found in the environment and in wound swabs is shown in Fig. 3. As the sources of Gram-negative organisms in the environment became fewer, a smaller proportion of wound swabs yielded Gram-negative organisms, but there was no change in the proportion of swabs yielding *Staphylococcus aureus*.

The endemic strain of *Pseudomonas aeruginosa*, serotype 10, phage pattern (100 RTD) 44/68+ was grown from a scrubbing-up tap in April, the floor surface

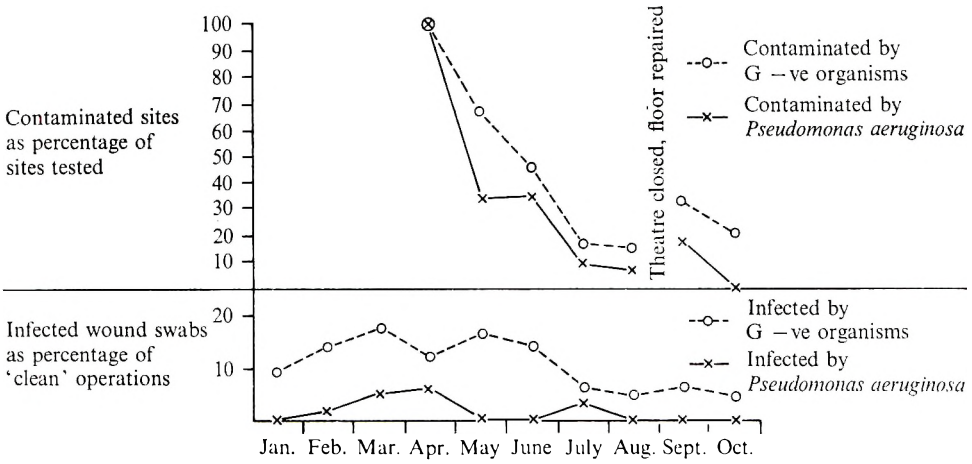

Fig. 3. Correlation between contaminated sites in operating theatre and infections in 'clean' incisional wounds.

in May and June, the floor mop in June, the floor outlet and the scrubbing machine tank (out of use) in September. The same strain was grown from one wound swab in March and two in April. The remaining four patients' strains were not typed.

DISCUSSION

It should not be accepted that a heavily contaminated environment is inevitable in an old hospital building nor that any improvement in hygienic standards will be costly. The part played by age and dilapidation in this case was limited, although there were serious failures of maintenance. Major faults lay in poor design or unsuitable choice of comparatively new equipment, and in failure to appreciate the nature of the risk of infection in various practices. Improving conditions in the operating theatre was not simple. Communication between members of the staff was incomplete, advice was not always understood or carried out and there had to be some changes of plan. Ayliffe, Brightwell, Collins & Lowbury (1969) found frequent discrepancies between methods thought to be in use and what was actually done in hospital wards, and they advocated systematic training in the control of infection at several staff levels.

The present work was not planned in advance as a scientific study, but arose from the practical need to reduce wound infection and was co-ordinated by the Control of Infection Committee. The entire co-operation of the hospital secretary, pharmacist, surgical teams, theatre sister and staff and maintenance staff, and reliable advice received informally from many outside quarters combined to achieve worthwhile improvements.

The aims of this work, which were to reduce the number of infections and to improve working conditions in the theatre were achieved, and at comparatively small cost, which was probably soon covered by a saving on antibiotics and treatment, since an excess stay in hospital of 7·3 days for patients with sepsis has been estimated (P.H.L.S. Report on surgical wound infection, 1960). The bacteriological

survey was neither detailed nor costly. It was carried out amid the routine of a small hospital laboratory, except for the typing of pseudomonas strains which was undertaken by the Cross-Infection Reference Laboratory, Colindale. When cost is being considered, it is worth noting that expensive equipment may be worse than cheaper methods. A new scrubbing machine was dirtier than mops, and an elaborate 'sterile' water apparatus in use was contaminated. In 1959, a Medical Research Council Working Party, and in 1964, Kelsey & Beeby, emphasized that water for use in operations should be autoclaved in bottles.

Gram-negative bacteria have often been demonstrated in hospital sink outflows, and careful observers have come to different conclusions. Kohn (1966) associated *Ps. aeruginosa* in outflows with infected burns, whereas Jellard & Churcher (1967) in a neonatal unit, and Lowbury *et al.* (1970) in a study of tracheostomies, concluded that patients were seldom infected from this source. Wormald (1970), also in a burns unit, found concordance of patient and basin outflow pseudomonas strains so infrequent that he discontinued a waste-trap sterilizing procedure. But he noted that a serotype 10 imported in a patient became firmly established in the wash-basin. Simple splash tests indicated that a danger exists if a susceptible victim and an infective dose of pathogens coincide. Bacteria from the bottom of a sink can splash onto a surgeon's gown and also enter and colonize scrubbing-up taps. Plug outlets here have been dosed with neat Hycolin at the end of each day. This method is expensive but has shown considerable success. Contamination of the washing taps was reduced by daily injection of hypochlorite solution. In addition the water supply tanks were put in order and the hot water temperature increased. The neglect of the tanks exemplifies a common failure to inspect and maintain old installations in hospitals. However, hospital plumbing design needs radical re-thinking to reduce the hazards of stagnant water.

Gross contamination of the theatre suite was traced to a modern scrubbing machine which provided a breeding ground for Gram-negative organisms and sprayed bacteria around the theatre. Draughts between a broken south door and an open north window sometimes aided this dispersion. Spray could reach the sinks as shown in Fig. 2. It was impossible to clean or sterilize the inside of the scrubbing machine tank or, because of a projecting filter, to empty it completely. When Hycolin replaced a detergent in the tank, the heavy load of micro-organisms was too great for chemical disinfection. No disinfectant could be depended upon to render safe a machine of this design. Indeed, Gram-negative organisms may multiply in disinfectant solutions. Maurer (1969) has described an increase in the numbers of pseudomonads in some disinfectant solutions. The findings indicate that the hygienic design of scrubbing machines needs attention. Brushes, tanks and feedpipes should be easy to remove and sterilize. Internal surfaces should be smooth, pipes short and the whole easy to clean. Brush splash should not rise at a high angle from the floor. One machine was reconstructed to meet these requirements.

Although there was no evidence to show that autoclave failure contributed to wound infection in this hospital, it was disturbing to find that adequate written instructions in the use of the autoclave had not been given and that it was not working reliably although regularly inspected by the manufacturers.

Many patients included in this survey were given antibiotics before pus was sent to the laboratory. In the last decade there has been a steady increase in the use of ampicillin which tends to be prescribed for rises of temperature regardless of any bacteriological findings. No doubt this explains the failure to grow bacteria from some wound swabs (Table 1). It has been suggested that a rise in Gram-negative infections may be due to increased use of broad spectrum antibiotics (Johnstone, 1970). Hexachlorophane preparations, which were in use at the start of the survey, have been considered a potential source of Gram-negative infection (Collins & Deverill, 1971).

In spite of the high level of Gram-negative contamination in the operating theatre, most patients escaped overt infection. Several hundred operations were performed and only seven pseudomonas wound infections reached the laboratory. Three of the cultures were typed and showed the same phage pattern as the endemic strain in the theatre environment. Although this does not exclude sources of the same organism in food or wards, it makes theatre infection a serious possibility. Factors increasing liability to post-operative infection have been reviewed (McEwin, 1970). Pseudomonas infection was related to poor condition of the patient. Wormald (1970) found that patients with burns of over 30 % body surface became colonized much more often than did those with less injury. Our pseudomonas-infected patients were older (average 65 years) and more ill (4/7 with cancer) than others and their wounds discharged pus for longer (60 days). For comparison, the average age of a similar number of patients with wound infections in which staphylococci alone were isolated was 50 years, 1/7 had cancer and their wounds discharged for 19 days. In no case was *Pseudomonas aeruginosa* isolated in pure culture. A klebsiella, a streptococcus, three *Staphylococcus aureus* and three *Escherichia coli* were isolated in association. *Pseudomonas aeruginosa* may offer little risk to adult patients who are not old and frail or burned. Nevertheless, a theatre is better off without vermin, be they pigeons, cockroaches or bacteria. The recent reports by Powell & Rogers (1971) of a salmonella colonization of a suction pipe system in a premature baby unit and by the Peterborough Public Health Laboratory (1971) of an outbreak of *Ps. aeruginosa* infection in a genito-urinary ward are relevant warnings.

It is obviously impossible to prove cause and effect in a non-experimental setting or to draw firm conclusions from small figures, but the sequence of events here is suggestive. The reduction in contamination in the theatre was accompanied by a reduction in infection. The effect of the survey on methods and decisions was beneficial. Hospital administrators may welcome an opportunity to visit premises under discussion accompanied by an informed source of advice.

In this work exhortation achieved little, but demonstration of correct methods, photographs and bacterial cultures showing the contamination arising from failure to use correct methods made a strong impression on the staff. While a tolerable standard of hygiene was achieved, it is questionable whether this would be maintained without surveillance.

Although this report may appear depressing at first sight, the improvement in standards reflects great credit on all those responsible for the upgrading and in

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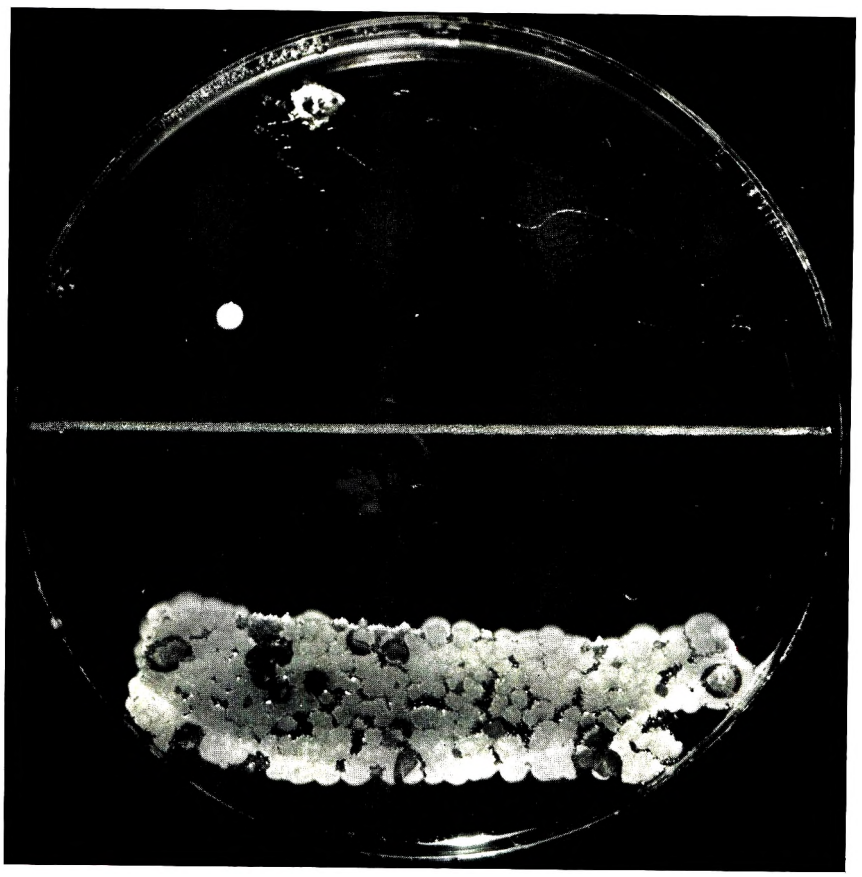

B

particular the members of the Control of Infection Committee. We hope that this description may encourage committees in other hospitals to examine critically, equipment and methods in use in operating theatres, not forgetting those used for cleaning. Mr Barfield's invaluable modification of a scrubbing machine, which it is planned to describe in detail elsewhere, is likely to play a significant part in the reduction of contamination in the environment of hospitals.

We are indebted to Dr J. C. Kelsey, Dr M. T. Parker and Dr T. M. Pollock for constructive criticism and also to some members of the Department of Health and Social Security for detailed technical advice. These include, in particular, Mr G. R. Wilkinson and Mr J. C. T. Williamson of the Scientific and Technical Services Branch of the Supplies Division, with Mrs J. Goodman and Mr I. W. Little of the Hospital Domestic Management Division.

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EXPLANATION OF PLATE

Two-swab plate (July 1970). A. Scrubbing machine brush (boiled). B. Scrubbing machine tank.

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Detection of *Bordetella pertussis* antibodies in human sera by complement-fixation and immunofluorescence

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SUMMARY

The complement-fixation test, as commonly used in the diagnosis of viral infections, was studied for its possible application to the diagnosis of whooping cough and the detection of antibody following pertussis vaccination. The results were compared with those obtained in parallel immunofluorescence tests. CFTs were performed on sera from 41 patients with whooping cough (*Bordetella pertussis* isolated), 125 vaccinated persons, and 618 controls; parallel tests by IF were made on sera from 24 cases of whooping cough, 36 vaccinated persons and 37 controls. Results of both tests correlated closely and showed that titres of diagnostic significance were seldom found in control sera. They also showed that, in patients suffering from whooping cough, antibody in a single specimen or a rise in antibody between paired sera was almost always demonstrated. Titres in general were lower in infants less than 6 months of age. IgG antibodies were involved in both tests. Although the number of sera tested was small both tests appear to be reliable as means of demonstrating the presence of antibody formed during the course of infection and after vaccination.

INTRODUCTION

The use of the complement-fixation test (CFT) as a means of indicating current infection has been found valuable in the diagnosis of bacterial, viral and parasitic infections. It has been used in whooping cough since its original performance by Bordet & Gengou (1906, 1907) but not routinely in diagnostic laboratories in recent years. As complement fixation (CF) is used in many laboratories today for the diagnosis of viral infections it seemed worth while to investigate results in known cases of whooping cough and in persons whose vaccination history was known. In addition it was decided to examine a proportion of these sera by immunofluorescence (IFT) in order to compare results with those obtained in parallel CFTs, and to attempt to identify the class of immunoglobulin involved in the fixation of complement.

MATERIALS AND METHODS

CFTs were performed on a total of 827 sera from 784 persons as follows:

(1) Forty-one patients with whooping cough from whom *Bordetella pertussis* was isolated;

(2) two groups of children in good health who had been vaccinated:

(i) Seventy-eight children under 5 years of age who had been given a primary vaccination course and a booster 18 months later (vaccine used not recorded). Sera were obtained at the following times after the booster injection: five sera at 4–5 months, 24 at 1 year, 23 at 2 years, 19 at 3 years, seven not known;

(ii) Forty-seven children under 2 years of age who were receiving a course of primary vaccination and booster with one of two different vaccines: 16 children were given vaccine A, which was Trivax containing aluminium hydroxide; 31 children were given vaccine B which was plain Trivax. Both vaccines were prepared by Wellcome Research Laboratories, Beckenham. During the course of vaccination random specimens were obtained according to availability. These might be either before or after primary vaccination or before or after the booster. It was not possible to obtain the whole series of specimens from any individual. A single specimen was obtained from 38 children, two specimens from eight and three from one child. Six and 32 sera were taken before and after vaccination respectively, and 8 and 11 before and after a booster respectively.

All sera in Group 2 (i) and (ii) were tested under code.

(3) Six hundred and eighteen persons aged 6 months to more than 80 years as controls: 147 were under investigation for hydatid disease; 34 for leptospiral infection; the rest were believed to be in good health.

IFTs were performed in parallel with CFTs on 24 sera from Group 1, 36 from Group 2(ii), and 37 from Group 3.

Six hundred and thirty-five specimens of sera received wet were stored either at 4° C. for periods up to 24 hr. or at −20° C. until the day they were tested. One hundred and ninety-two specimens received freeze-dried were stored at 4° C. until they were reconstituted in distilled water on the day of testing. All sera were heated at 56° C. for 30 min. immediately before the CFT was performed. All titres throughout this paper are expressed as reciprocals. Doubling dilutions of sera ranging from 10 to 640 were tested by CF. In IFTs doubling dilutions of 5–20 were tested initially, and further dilutions were tested when no end-point had been obtained.

Preparation of pertussis antigens

For complement-fixation tests

Strains GL 353 (Type 1), BT 2 (Type 1, 3), 360 E (Type 1, 2), LN 16 (Type 1, 3; mainly 3), kindly supplied by Dr N. W. Preston of the University of Manchester, were used for the preparation of antigens by a method based on that described by Weichsel & Douglas (1937).

A 48-hour growth of *B. pertussis* on charcoal blood agar plates (85 mm. in diameter) was washed off each into 2 ml. sterile physiological saline. After shaking

vigorously for a few minutes, the suspension was heated for one hour at 60° C. It was then centrifuged at about 3000 rev./min. for 20 min. The slightly opaque supernatant fluid was used as an antigen. Sodium azide (0·08 % final concentration) was added as preservative. Antigens remained potent for at least 6 months when stored at 4° C.

For immunofluorescence tests

The suspension of killed *B. pertussis* organisms which had been prepared for agglutination tests on diagnostic antisera raised in rabbits, was used as antigen. It was made in 1963 from strains L92 and 18323K (Type 1, 2 and 4) and 3747 (Type 1, 2, 5 and 6) kindly supplied by Dr A. F. B. Standfast of the Lister Institute of Preventive Medicine, Elstree. Briefly, the suspension consisted of whole bacterial organisms which were grown on plates of Bordet Gengou agar for 3 days and then washed off into 0·25 % buffered formol saline containing glass beads. The suspension, with beads, was placed on a shaker overnight to break up any clumps of organisms. The suspension was then washed to remove the formalin. The organisms were finally resuspended in saline containing 1/10,000 merthiolate to a concentration equivalent to about 2×10^{10} *Escherichia coli* organisms per ml., and were stored thereafter at 4° C.

Serological tests

The complement-fixation test

All CFTs were performed by the method described by Bradstreet & Taylor (1962). Three HD 50 of complement were used; tests were incubated overnight at 4° C. before the sensitized sheep cells were added. Antigens were standardized in chessboard titrations with individual sera from cases of whooping cough obtained during convalescence and from these the optimal dilution of each antigen for use in single line serum titrations was selected. At first, antigens prepared from all four strains were used to test sera but because results were very similar, subsequent tests were made with only two antigens, BT 2 and LN 16. Titres with each never varied by more than one dilution; the highest titre of a serum obtained with either antigen was the one recorded.

The immunofluorescence test

The IFT was performed as described by Edwards, Tannahill & Bradstreet (1970) but started with a dilution of 1/5 of the human serum and the stored agglutinating antigen was diluted 1/10. The conjugate was used at its optimal dilution, i.e. 1/10.

A swine antihuman IgG FITC conjugate prepared by Nordic Pharmaceuticals was used. This conjugate is prepared by immunizing swine with purified IgG from pooled normal human serum. Purified IgG is then extracted from the swine serum and conjugated with fluorescein. Although not absorbed with light chain components the reaction with other human immunoglobulins is not great.

A pool of human serum from known convalescent cases found strongly positive by IF, and a pool of human serum from sera previously tested by IF and found to be negative at a dilution of 1/5, were included as serum controls.

RESULTS

The results of CFTs on the 618 control sera are presented in Table 1. Less than 7 % of these sera had titres > 10.

Results of CFTs on all sera from 41 known cases of whooping cough are shown in Table 2. This table also shows, where known, the age of the patients and the time since onset of the infection when the first specimens were taken. Two specimens of sera were obtained from 33 of the patients. The period after onset of disease when first specimens were collected was reported for 10 patients only, and ranged from 7 to 48 days. The time of collection of second specimens of sera (not shown in the table) was usually 2–3 weeks after the first. It can be seen that titres in general were lower in infants under 6 months of age than in older patients. Table 3 shows a comparison of titres obtained in the paired sera: of the 33 pairs, only 9, taken from patients who in Table 2 were numbered P 2, P 3, P 9, P 11, P 12, P 14, P 16, P 23 and P 25, did not in either specimen show a titre of antibody > 10; 7 of these pairs came from children of 4 months of age or less, and the remaining two from two children under 1 year. Where single specimens only were tested 5/8 had antibody titres > 10.

No table is presented to show the results on the sera from 78 children in Group 2(i) because antibody was detected in only two specimens; one from a child 1 year after boosting had a titre of 10, and the other 2 years after boosting a titre of 20; all other sera were < 10.

Table 4 shows the results of CFTs on sera from children in Group 2(ii): traces of antibody at a dilution of 1/10 were found in 2/6 sera from children before immunization with vaccine B; no antibodies were found in eight pre-booster specimens; all sera from children vaccinated with either two or three injections of vaccine A showed an antibody response which ranged from 10 to > 80, but there was a poor antibody response in sera from children immunized similarly with vaccine B for 7/18 had titres of < 10 and a further seven had a titre of 10 only; antibody was found in sera from 4/4 children following a booster injection with vaccine A, but in only 5/7 children after a booster injection with vaccine B. However, the two negatives were taken at 12 and 24 weeks and it appears that titres were lower after the 10th week.

A comparison of results obtained by CF and IF is shown in Table 5. Because in the CFT 83 control sera (13·4 %) gave titres of 10 (Table 1), and two pre-vaccinated

Table 1. *Complement-fixation tests on human control sera*

	Serum titres					
Age (years)	< 10	10	20	40	80	Total
0–5	103	20	9	—	1	133
6–15	25	14	6	—	—	45
16–40	278	34	15	6	—	333
> 40	62	5	3	—	—	70
Not known	26	10	—	1	—	37
Total	494	83	33	7	1	618
%	80	13·4	5·4	1·1	0·1	100

Table 2. *Results of CF tests on sera from known cases of whooping cough*

Patient no. P	Age Months	Days since onset	Paired sera 1st specimen	Paired sera 2nd specimen	Single specimen
1	1	—	10	20	—
2	1	—	< 10	< 10	—
3	2	—	< 10	< 10	—
4	2	—	—	—	10
5	2	—	—	—	20
6	2	—	< 10	20	—
7	3	—	10	80	—
8	3	—	< 10	40	—
9	3	—	< 10	< 10	—
10	3	—	< 10	20	—
11	3	—	< 10	10	—
12	3	—	< 10	10	—
13	3	—	—	—	< 10
14	4	14	< 10	< 10	—
15	4	42	—	—	40
16	4	—	< 10	< 10	—
17	4	—	< 10	20	—
18	6	—	10	80	—
19	6	—	10	20	—
20	8	—	—	—	< 10
21	9	—	10	40	—
22	10	31	—	—	80
23	10	—	< 10	10	—
24	11	—	40	80	—
25	11	—	< 10	10	—
	Years				
26	1	48	40	160	—
27	1	—	40	40	—
28	1	28	—	—	20
29	1	—	40	320	—
30	2	—	160	160	—
31	2	—	< 10	80	—
32	2	—	< 10	40	—
33	3	11	< 10	80	—
34	3	10	80	40	—
35	3	—	< 10	40	—
36	3	—	40	40	—
37	6	7	10	80	—
38	11	—	< 10	40	—
39	11	—	10	40	—
40	Adult	21	—	—	40
41	Not known	14	80	160	—

Table 3. *Comparison of titres in paired sera from* 33 *patients*

⩾ 4-fold rise	16
2-fold rise	4
No change: > 10 in both	3
Fall: > 10 in both	1
No change: ⩽ 10 in both	9
Total	33

Table 4. *Results of CFTs on sera from children during their primary course of injections*

Serum no.	Vaccine	No. of injections	Pre-vaccination	Post-vaccination CF titre	Weeks*	Pre-boost titre	Months*	Post-boost titre	Weeks*
1*a*, 1*b*	A	2	—	20	3	< 10	8	—	—
2	A	2	—	⩾ 80	4	—	—	—	—
3*a*, 3*b*	A	2	—	20	8	< 10	12	—	—
4*a*, 4*b*	A	2+Bo	—	10	16	—	—	40	5
5	A	3	—	⩾ 40	4	—	—	—	—
6*a*, 6*b*	A	3+Bo	—	⩾ 40	4	—	—	40	7
7	A	3	—	40	4	—	—	—	—
8	A	3	—	10	6	—	—	—	—
9	A	3	—	80	6	—	—	—	—
10	A	3	—	20	8	—	—	—	—
11	A	3	—	20	9	—	—	—	—
12	A	3	—	20	9	—	—	—	—
13*a*, 13*b*	A	3+Bo	—	40	9	—	—	40	6
14	A	3	—	10	15	—	—	—	—
15	A	3+Bo	—	—	—	—	—	10	10
16	A	3	< 10	—	—	—	—	—	—
17*a*, 17*b*	B	2	tr 10	< 10	4	—	—	—	—
18*a*, 18*b*	B	2+Bo	—	< 10	5	< 10	11	—	—
19	B	2	—	10	9	—	—	—	—
20	B	2	—	tr 10	10	—	—	—	—
21*a*, 21*b*, 21*c*	B	2+Bo	< 10	< 10	12	—	—	< 10	12
22*a*, 22*b*	B	2+Bo	—	< 10	14	< 10	11	—	—
23	B	3	—	20	4	—	—	—	—
24	B	3	—	10	4	—	—	—	—
25	B	3	—	40	4	—	—	—	—
26	B	3	—	< 10	5	—	—	—	—
27	B	3	—	10	5	—	—	—	—
28	B	3	—	< 10	5	—	—	—	—
29	B	3	—	10	6	—	—	—	—
30	B	3	—	10	6	—	—	—	—
31	B	3	—	10	7	—	—	—	—
32	B	3	—	tr 10	8	—	—	—	—
33	B	3	—	< 10	13	—	—	—	—
34	B	3	—	80	13	—	—	—	—
35	B	3	—	—	—	< 10	7	—	—
36	B	3	—	—	—	< 10	8	—	—
37	B	3	—	—	—	< 10	9	—	—
38	B	3	—	—	—	< 10	10	—	—
39	B	3+Bo	—	—	—	—	—	80	4
40	B	3+Bo	—	—	—	—	—	20	4
41	B	3+Bo	—	—	—	—	—	10	5
42	B	3+Bo	—	—	—	—	—	20	11
43	B	3+Bo	—	—	—	—	—	20	18
44	B	3+Bo	—	—	—	—	—	< 10	24
45	B	3	< 10	—	—	—	—	—	—
46	B	3	< 10	—	—	—	—	—	—
47	B	3	tr 10	—	—	—	—	—	—

* = time since last injection; Bo = booster; tr = trace reading.

children gave trace readings at 10 (Table 4), in this test a titre > 10 in a single specimen was accepted as one of diagnostic significance; by IF it was taken as five. Results were said to agree when, in a single specimen, both tests showed either no antibody or a significant titre as defined above and, in paired specimens, when both tests showed a rise in titre of four-fold or greater. In 88/97 tests there was agreement: 30 of these were positive and 58 were negative. Four sera which showed titres of 20 in IFs were negative in CFTs. Tests were repeated and CFTs remained negative even when performed in chessboard titrations with dilutions of antigen commencing at 1/2.

DISCUSSION

In agreement with other reports our results show that CF antibodies to *B. pertussis* are found less frequently in infections which occur during the first months of life (a Combined Scottish Study, 1970) and that they appeared in the majority of patients during or shortly after an infection (Weichsel & Douglas, 1937; Wilson & Miles, 1964). Thus, in our studies, at the time the second specimen of serum was taken 24 of all 33 patients and 18/20 patients who were of 6 months of age or more had antibody titres > 10, and among single specimens tested the 3 with titres ⩽ 10 were from patients under 1 year. Although sera from cases were not tested serially to study any change in titres during late convalescence and beyond, it is likely that antibodies would ultimately disappear since the majority of results with control sera were negative. Control sera with titres > 10 may have come from persons already immune whose antibodies had been boosted following recent contact with a case of whooping cough.

From the close correlation of results in parallel tests by CFT and IF (Table 5) it is shown that the antibodies involved in CF are of class IgG. The positive titres obtained by IF which were < 10 by CFT may reflect antigenic variation in the pertussis strains used in the preparation of the suspension since these were different from the ones used to prepare the CF antigen, or possibly the existence in the sera of different IgG components. The discrepancies do not appear to represent simply a difference in the sensitivities of the two tests because the levels of antibody titres in each, where significant, did not always run parallel.

The vaccine used to immunize the 78 children in Group 2(i) was different from those used for the children in Group 2(ii) and the titres of CF antibody present, if

Table 5. *Serum antibody titres in CF and IF tests performed in parallel*

Source of sera	No. of sera	Agree	IF ⩾ 5 CF ⩽ 10
Known positive	24	18	6*
Vaccinated	36	33	3†
Control	37	37	0
Total	97	88	9
%	100	90·7	9·3

* Two sera with an IF titre of 5, one of 10, and three of 20.
† Two sera with an IF titre of 5, and one of 20.

any, in their early post-booster sera were not known. It was not possible, therefore, to interpret with certainty the finding of negative results in the 76/78 sera tested by us. However, it was this unexpected observation, especially in sera taken only 4 to 5 months after the booster injection, that initiated the investigation on the sera from children in Group 2(ii). From this Group an attempt was made to find out if CF antibody was at any time provoked by vaccination and, if so, how long it remained present. Although the number of children tested was small and sera were never obtained from any single child throughout the course of injections, we, nevertheless, feel able to draw a few tentative conclusions from the limited findings observed.

Significant titres of CF antibody are not detectable before vaccination; they are provoked to a greater or lesser extent by different vaccines, and 2 injections with a potent vaccine may provoke more antibody than three with a less potent one; CF antibodies are not detectable 7 months, or possibly sooner, after the primary course of vaccination and they reappear following a booster; lastly, the time of disappearance of antibodies after the booster injection is not known, but it may be as early as 4 to 5 months if the negative results obtained in the 78 children in Group 2(i) represents a fall in titre. These conclusions must, of course, be subject to confirmation by serial investigations on a larger number of children.

The manufacturers of vaccines A and B used in these studies stated in a personal communication that 'in six mouse protection tests the best estimates of potency was that vaccine A had a potency of 12 protective units per dose of vaccine and vaccine B had four'. This assessment correlates well with the titres of CF antibodies found in the children in Group 2(ii) given the two vaccines.

In general, antibodies of class IgM are known to agglutinate corresponding antigens more readily than those of IgG. The agglutination reaction was the test used in a series of investigations made by the Whooping Cough Immunization Committee of the Medical Research Council (Report, 1959) on the prophylactic value of vaccines and their assessment by a laboratory test. They showed that sera from mice immunized with an antigenic extract (Pillemer, Blum & Lepow, 1954) contained low titres of agglutinating antibody compared with those immunized with some whole bacterial vaccines, although the Pillemer antigen and bacterial vaccines both showed good protection in children. From this it was concluded that agglutinin production in mice could not always be taken as evidence of protective activity of a vaccine in children. Since then the assessment of protective activity of a vaccine in the laboratory has been made by means of the mouse protection test. If the Pillemer antigen – an antigenic extract – provoked mainly IgG antibodies these would agglutinate bacterial suspensions less efficiently than IgM, and this fact may account for the poor response found in mice which were vaccinated with Pillemer antigen. A CFT performed in these circumstances might have detected such IgG antibodies in mice as efficiently as the agglutination reaction detected IgM antibodies formed after vaccination with whole bacterial organisms. If it can be shown that CF titres in mice after vaccination correlate with protection in children, as CF titres in children appear in these studies to correlate with protection in mice, then it might be possible to use this test routinely for assessing the

potency of a vaccine in place of the mouse protection test which is more laborious and costly. Investigations are in progress to study this point.

Agglutination reactions for diagnosis were not made in this study because in our experience smooth stable killed suspensions can be difficult to prepare, and they may not be as sensitive in tests with human sera as suspensions of living organisms (Evans & Maitland, 1939). The extract used in the CFT reported here is easy to prepare, and it remains potent and free from anticomplementary activity when stored at 4° C. for long periods. The use of the CFT routinely in cases of suspected whooping cough in which an organism has not been isolated will provide a reliable indication in retrospect of infection due to *B. pertussis*, particularly in patients over 6 months of age. The use of IF as an alternative test would appear to be equally satisfactory.

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Membrane antigens of *Mycoplasma hominis*

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SUMMARY

Extraction of membranes of *Mycoplasma hominis* with *n*-butanol showed that antigenicity was associated with the non-lipid residue, which probably consisted mainly of protein, and not with the lipid itself.

Since many membrane proteins are hydrophobic, membranes were rendered soluble in various ways. Extraction with urea or phenol was the most successful, yielding extracts which were both antigenic and serologically reactive. The urea extract could not be fractionated by polyacrylamide disk electrophoresis or by column chromatography. However, serologically active components identified by gel diffusion were separated from detergent-lysed membranes by polyacrylamide disk electrophoresis. The activities of antisera against these fractions suggested that indirect-haemagglutinating or metabolic-inhibiting antibodies can be directed against several different membrane antigens. However, the antigens identified by gel diffusion probably do not represent all the components participating in indirect haemagglutination.

Treatment of membrane suspensions with heat, alkali, periodate and various enzymes showed that the four components identified by gel diffusion could be distinguished by their differing stabilities and properties. On the basis of their lability and susceptibility to proteolytic enzymes, two were identified as proteins.

INTRODUCTION

The membrane antigens of *Mycoplasma hominis* are important in indirect haemagglutination (IHA), metabolic inhibition (MI) and growth inhibition (GI) (Hollingdale & Lemcke, 1969) and as receptors for the adsorption of H-HeLa cells (Hollingdale & Manchee, 1972). The membrane is also involved in complement fixation (CF) but, in addition, antigens from the soluble cell contents (soluble fraction) participate in this reaction. Chemical extraction of whole organisms shows that serologically reactive extracts are obtained only by methods known to extract proteins. Lipids are only weakly active and aqueous phenol extracts for carbohydrates completely inactive. Since membranes are so important to the antigenicity of the cell and selective extraction of membrane antigens from whole cells by chemical methods was not possible (Hollingdale & Lemcke, 1969), membranes

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were prepared and an attempt made to separate and characterize the serologically active membrane components. Some of these results were briefly reported at the Fifth International Congress of Infectious Diseases (Lemcke & Hollingdale, 1970).

MATERIALS AND METHODS

Preparation of membrane suspensions

Membranes were prepared by ultrasonic treatment and differential centrifugation from suspensions of *Mycoplasma hominis* strain SC4, grown, harvested and washed as described by Hollingdale & Lemcke (1969). Membranes sedimented at 100,000 *g* were washed six times in dilute sodium chloride/tris buffer (0·0075 M sodium chloride, 0·0025 M tris, pH 7·4) before resuspension in the same buffer at 5–6 mg. protein/ml. and storage at $-30°$ C. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). All preparations were tested by gel diffusion for the absence of components from the soluble fraction using antiserum to whole organisms, the membranes lysed with Triton X-100, and a soluble fraction prepared by freezing and thawing (Hollingdale & Lemcke, 1969).

Serological tests

Gel diffusion, metabolic inhibition and indirect haemagglutination tests were carried out as previously described (Hollingdale & Lemcke, 1969), except that growth medium (10%, v/v) was included in the gel diffusion agar when testing antisera to the various membrane preparations (see below), and for indirect haemagglutination the unit volume was reduced to 0·025 ml. In gel diffusion, all antigens were tested at 2 mg. protein/ml. and membranes were lysed with Triton X-100 at 5 mg./mg. membrane protein.

Preparation of 'lipid-free' membranes

The method was based on that of Rodwell, Razin, Rottem & Argaman (1967). Five volumes of membrane suspension in dilute sodium chloride/tris buffer at 5 mg. protein/ml. were extracted with two volumes of *n*-butanol in an ice-water bath for 30 min. The butanol layer was separated by centrifugation at 30,000 *g* for 20 min. The insoluble pellet and interfacial material was resuspended in the aqueous layer, re-extracted with two volumes of butanol and centrifuged as before. The pooled butanol layers and the aqueous phase were separately dialysed against buffer. The lipid was then dispersed in the buffer by sonication at 20 kc. per sec. for 2 min. (Branson S75 Sonifier, Branson Instruments Inc., Stamford, Connecticut, U.S.A.). The pellet of lipid-free membranes was washed three times and resuspended in buffer to the volume of the original membrane suspension.

For 'recombined' membranes, half the final volume of the lipid extract and the lipid-free membrane suspension were mixed and dialysed for 18 hr. at 4° C. against dilute sodium chloride/tris buffer containing 0·05 M magnesium chloride. Assuming no loss during fractionation, the lipid and lipid-free material in the recombined membranes should have been present in the same proportion as in the original membranes.

Preparation of soluble protein extracts

The methods of Maddy (1966) and Zwaal & van Deenen (1968) for preparing soluble protein or lipoprotein extracts from mammalian erythrocyte ghosts were used with *M. hominis* membrane suspensions. Treatment with lithium chloride or lithium bromide at concentrations from 0·1 to 8 M was also tried. For urea extraction, equal volumes of membrane suspension (2 mg. protein/ml.) and 16 M urea in sodium chloride/tris buffer were held at room temperature for 30 min. with occasional shaking. The insoluble residue was deposited by centrifugation at 8000 *g* for 30 min. and the supernatant dialysed successively against 4 M and 2 M urea in buffer and buffer alone for 3, 18 and 24 hr. respectively at 4° C. Before concentration by pressure dialysis, the absence of urea was checked with urease tablets and Nessler's reagent (British Drug Houses, Poole, Dorset). Extraction with phenol was carried out by the method used for whole organisms (Hollingdale & Lemcke, 1969).

Gel filtration and polyacrylamide disk electrophoresis of urea extract

A column (1·7 × 38 cm.) of Sephadex G-50 (fine; Pharmacia AB, Uppsala, Sweden) was equilibrated with sodium chloride/tris buffer (0·015 M sodium chloride, 0·05 M tris) pH 7·4. Urea extract (2 ml. containing 4 mg. protein) was layered onto the column and eluted with the buffer used for equilibration. Fractions were collected at 3 min. intervals and monitored for absorbance at 280 nm.

The method of polyacrylamide disk electrophoresis was that of Davis (1964) except that the spacer gel was omitted and the sample containing 750 μg. of protein was layered onto the gel in sucrose (20 %, w/v).

Absorption of antiserum

Lipid-free and recombined membranes and the phenolic and urea extracts were tested for their ability to remove MI and IHA antibody from antiserum to whole organisms by absorption of 0·1 ml. of antiserum with extracts containing 1 mg. of protein (Hollingdale & Lemcke, 1969).

Separation of membrane proteins by polyacrylamide disk electrophoresis

The method was based on that of Maizel (1966). Membranes were lysed with sodium dodecyl sulphate (SDS) or Triton X-100 to give final concentrations of 2 mg. membrane protein and 20 mg. detergent/ml. Two volumes of lysate and one volume of 60 % sucrose were mixed and 0·15 ml. (containing 200 μg. protein) was layered onto each gel. Gels consisted of acrylamide (5 %, w/v) with *N*,*N*′-methylenebisacrylamide (0·13 %, w/v), and SDS (0·1 %, w/v) or Triton X-100 (0·1 %, w/v) in 0·1 M sodium phosphate, pH 7·0. Polymerization was catalysed by *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (0·05 %, v/v) and ammonium persulphate (0·075 %, w/v). Where gels were to be used for inoculation of rabbits, ammonium persulphate was removed by electrophoresis for 2 hr. at 5 mA per gel before applying the lysed membranes. Electrophoresis of detergent-lysed membranes was carried out for 2 hr. at 8 mA for each gel in 0·1 M phosphate buffer, pH 7·2, using a Shandon electrophoretic apparatus with 75 × 5 mm. tubes.

Some gels were stained to demonstrate protein bands with naphthalene black (1 %, w/v) in acetic acid (7 %, v/v). To locate serologically active bands by gel diffusion, gels were sliced transversely into 3 mm. sections. With a syringe, sections were macerated into alternate wells 5 mm. in diameter and 3 mm. distant from a central 60 × 1 mm. trough. The wells containing macerated sections were filled with Triton X-100 (4 %, v/v) to elute the membrane components. This was used in preference to SDS in tests with antisera, because SDS gives a non-specific precipitate with rabbit serum. The remaining wells were filled with membranes lysed with Triton X-100 and the trough with antiserum to whole organisms. Diffusion was at room temperature. Since eight gels could be run at one time and only two were required for protein staining and location of the serologically active proteins, the other gels were sliced into 3 mm. transverse sections which were numbered from the origin. Sections with the same number were pooled, immediately frozen and stored at −30° C. for future use as rabbit inocula. Altogether eight electrophoretic runs were made and the sections containing the different components identified. Before inoculating rabbits, sections from each run were retested for gel diffusion activity.

Antisera

These are listed in Table 1. Antisera to whole organisms and to purified membranes of SC4 were those prepared by Hollingdale & Lemcke (1969). For antisera to lipid-free or recombined membranes, preparations were emulsified in adjuvant (Esso Markol 52 : Arlacel; 9 : 1 v/v) and given in two subcutaneous (s.c.) inoculations separated by an interval of 13 days. Three further intravenous (i.v.) injections of an aqueous suspension were given 13, 15 and 17 days after the second s.c. inoculation. A total of 4 mg. of protein was injected. A similar schedule was followed for antiserum to the urea extract, of which 5 mg. of protein was inoculated.

For antisera to membrane proteins separated by polyacrylamide disk electrophoresis, suitable batches of 16–19 gel sections, which contained the same precipitating component, were homogenized in saline and emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, U.S.A.). Half of each emulsion was given subcutaneously on the inside of the right hind leg and half intramuscularly into the left flank. After 14 days, a similar emulsion was

Table 1. *Preparations of* Mycoplasma hominis *SC4 against which antisera were prepared*

Whole organisms
Membranes purified on sucrose density gradients (purified membranes)
Lipid-free membranes (butanol-extracted)
Recombined membranes (lipid-free membranes and butanol-extracted lipid)
Urea extract of membranes
Phenolic extract of membranes
Membranes lysed with sodium dodecyl sulphate
Membrane components separated by polyacrylamide disk electrophoresis:
  Antiserum A, against precipitating component 1
  Antiserum B, against precipitating component 3
  Antiserum C, against precipitating components 2*a*, 2*b* and 3
  Antiserum D, against precipitating components 2*a*, 2*b* and 3

inoculated by the same routes in collateral positions. Rabbits were bled 7, 14, 21 and 28 days after the first inoculation. A similar schedule was followed for antisera against SDS-lysed membranes, of which the rabbits received 1·6 mg. protein, and the phenolic extract, of which 2 mg. was inoculated.

Specificity of antisera

All the antisera in Table 1 were tested by gel diffusion against the growth medium, human serum and human γ-globulin (Lister Institute, Elstree, Herts.), either alone or with Triton X-100 at the concentration used for membranes. Antisera were also tested by gel diffusion against a soluble cell fraction of *M. hominis* prepared by freezing and thawing.

Enzymes

These were obtained from the following sources: pronase (B grade), Calbiochem, Los Angeles, U.S.A.; trypsin (ex bovine pancreas, 1x crystallized, 8000 BAEE units/mg.), lipase (ex hog pancreas), trypsin inhibitor (soybean) and β-glucosidase

Table 2. *Treatment of membranes of* Mycoplasma hominis *SC* 4

Agent	Final concentration	Time	Buffer	Test for enzyme activity
Heat (50°, 60°, 80°, 100° C.)	—	30 min.	A	—
Sodium meta-periodate	0·2 M	24 hr. (dark)	A	—
Sodium hydroxide	0·1 M	1 hr.	A	—
Trypsin	0·1 mg./ml.	2 hr.	B	Gelatin agar (Barer, 1946)
Pronase	0·1 mg./ml.	2 hr.	C	Gelatin agar (Barer, 1946)
Lipase	1·0 mg./ml.	16 hr.	C	Tributyrin agar (Willis, 1965)
α-Glucosidase	1·0 mg./ml.	16 hr.	C	Liberation of glucose from *p*-nitrophenyl α-D-glucoside or *p*-nitrophenyl β-D-glucoside
β-Glucosidase	1·0 mg./ml.	16 hr.	C	Liberation of glucose from *p*-nitrophenyl α-D-glucoside or *p*-nitrophenyl β-D-glucoside
α-Galactosidase	82 EU/ml.	16 hr.	C	Liberation of galactose from *p*-nitrophenyl α-D-galactoside or *o*-nitrophenyl β-D-galactoside
β-Galactosidase	3·4 EU/ml.	16 hr.	C	Liberation of galactose from *p*-nitrophenyl α-D-galactoside or *o*-nitrophenyl β-D-galactoside
Phospholipase A	0·1 mg./ml.	30 min.	D	Hydrolysis of egg yolk
Phospholipase C	1 EU/ml.	30 min.	E	Hydrolysis of egg yolk

Buffers: A 0·0075 M sodium chloride/0·0025 M tris, pH 7·4.
B 0·05 M tris, pH 7·8.
C 0·1 M phosphate, pH 7·0.
D 0·05 M tris, pH 7·2.
E 0·05 M tris/0·05 M Ca^{2+}, pH 7·2.

Sodium metaperiodate treatment at room temperature, sodium hydroxide and enzymes at 37 °C.

(ex sweet almonds), Koch-Light Laboratories Ltd, Colnbrook, Bucks.; β-galactosidase (ex *Escherichia coli*), Boehringer Corporation, London, W.5. Phospholipase A (crystalline, ex cobra venom) and phospholipase C (*Clostridium welchii*) were kindly supplied by Dr G. M. Gray and α-galactosidase (*Trichomonas foetus*) by Professor W. M. Watkins of this Institute.

Treatment of membrane suspensions

The details are summarized in Table 2. Membranes were treated at 3–4 mg. protein/ml. In every experiment a control membrane suspension was held with buffer under the same conditions. Membranes held at room temperature for 30 min. served as the control for heated membranes. Treated and control membranes lysed with Triton X-100 were tested by gel diffusion, without removal of the agent, against antisera to whole organisms and to membrane components separated in polyacrylamide gels. In this way inactivated components could be identified.

The capacity of the treated membranes to adsorb MI and IHA antibody from antiserum to whole organisms was also determined where gel diffusion tests indicated that components had been destroyed. Antiserum previously inactivated and absorbed with washed sheep erythrocytes was mixed with membrane suspensions at a rate of 1 mg. protein to 0·1 ml. of serum and absorbed for 48 hr. at 4° C. Particulate matter was removed by centrifugation and the absorbed sera stored at −30° C. Heat-treated membranes were mixed directly with the serum, but membranes treated with sodium hydroxide or sodium periodate and the corresponding controls were first dialysed. In one experiment sodium hydroxide was neutralized with 0·1 N hydrochloric acid. Trypsin inhibitor was added to pronase or trypsin-treated membranes. In each experiment, a sample of antiserum was held with buffer as an 'unabsorbed serum' control, and was titrated at the same time as antiserum absorbed with treated or control membranes.

RESULTS

Reaction of detergent-lysed membranes in gel diffusion with whole organism antiserum

Three major precipitin bands, all apparently complex, were identified in reactions between detergent-lysed membranes of SC4 and antiserum to whole organisms. A fourth line sometimes appeared nearest to the antigen well (Hollingdale & Lemcke, 1970) but as its appearance was inconsistent, only the three major components were numbered, starting at the antiserum well. Components 1 and 3 sometimes appeared double, but, as component 2 was invariably double, the two lines were designated 2*a* and 2*b* (Pl. 1*a*). These precipitating components were distinct from those given by soluble fractions of SC4. These reactions were evidently specific to the membrane, since antiserum to whole organisms which had been raised to SC4 grown in rabbit serum medium (Hollingdale & Lemcke, 1969) did not react with any of the medium constituents or with γ-globulin (Pl. 1*a*).

Specificity of antisera to membrane preparations

Of the antisera listed in Table 1, only those against detergent-lysed, lipid-free or recombined membranes and the serum C, which contained antibody to components 2*a*, 2*b* and 3, reacted in gel diffusion tests with growth medium, human serum and human γ-globulin, with or without Triton X-100. All three gave reactions of identity with the antisera, showing that it was the γ-globulin constituent of the human serum in the growth medium which had been adsorbed to the membrane of *M. hominis*. Detergent-lysed membranes, however, did not react with antiserum to human serum (species-precipitating serum, Burroughs Wellcome, Beckenham, Kent) in similar tests. This suggests that only a small amount of γ-globulin adsorbed to the membranes, though sufficient to elicit a response in rabbits. All antisera which had been raised to membrane fractions or extracts were therefore absorbed with an equal volume of medium for 48 hr. at 4° C. before testing for IHA antibody. Since the MI test was in medium containing human serum, any antibody to human γ-globulin would be rapidly bound and absorption with medium was therefore unnecessary. For gel diffusion tests, growth medium (10 %, v/v) included in the agar was sufficient to prevent the formation of precipitin lines between membrane antisera and human serum or γ-globulin at 2 mg. protein/ml., and should therefore have blocked any reaction with the small amounts of γ-globulin contaminating detergent-lysed membranes. Antisera to the various membrane preparations did not react with soluble fractions in gel diffusion tests. Thus, reactions between these sera and detergent-lysed membranes should have been specific to the membrane constituents.

Serological activity and antigenicity of lipid-free and recombined membranes

Both lipid-free and recombined membranes gave rise to antisera with MI and IHA titres comparable to those of antiserum against membranes purified on sucrose density gradients (Table 3). Moreover, in gel diffusion tests these antisera reacted with detergent-lysed membranes to give the same complex of precipitin lines as whole organism antisera. Both preparations also absorbed MI and IHA antibody from antiserum to whole organisms to the same degree as untreated membranes (Table 3). In gel diffusion tests with whole organism antiserum, lipid-free and recombined membranes lysed with Triton X-100 gave only one line corresponding to component 1. However, since their antigenicity and absorption capacity was comparable to that of untreated membranes, this probably reflects the failure of the detergent to lyse the butanol-treated membranes rather than a loss of antigens. The butanol-extracted lipid neither reacted in gel diffusion tests nor absorbed MI or IHA antibody from antiserum to whole organisms (Table 3). Comparison of lipids in the butanol extract with those in untreated and butanol-extracted membranes by thin layer, silica gel chromatography suggested that at least 95 % of the lipid was removed by butanol (G. M. Gray, personal communication).

Serological activity and antigenicity of soluble protein extracts

Membrane extracts obtained by the methods of Maddy (1966) or Zwaal & van Deenen (1968) or with lithium salts contained only small amounts of protein and gave, at best, weak reactions in gel diffusion tests with antiserum to whole organisms. However, urea and phenolic extracts gave lines corresponding to components 1 and 3. When antiserum to whole organisms was absorbed with these extracts IHA titres were reduced from 2560 to $\leqslant 10$ ($\geqslant 256$-fold), although the corresponding reduction in MI titres was only 8–16-fold.

Antiserum to the urea extract contained antibody to component 3 but only very low concentrations of antibody to component 1, since it gave only a weak reaction with component 1 of detergent-lysed membranes (Pl. 1*b* and *c*) and no component 1 reaction with its homologous extract in gel diffusion. MI and IHA titres were also low (Table 4). Antiserum to the phenolic extract had MI and IHA titres comparable to those given by antiserum to purified membranes (Table 4) and gel diffusion tests showed that it contained antibody to components 1 and 3 (Plate 1*b* and *c*).

All the serologically active material in the urea extract was eluted from Sephadex G-50 in one peak immediately following the void volume and there was no separation. Similarly, no separation of proteins was obtained by polyacrylamide gel electrophoresis.

Activity of antiserum to membranes lysed with sodium dodecyl sulphate

Membranes were still antigenic after lysis with SDS. MI titres of the resulting antiserum were of the same order as those of antiserum to purified membranes (Table 4). However, IHA titres were eight-fold lower, although adjuvant was used in the preparation of this antiserum whereas serum to purified membranes was produced by i.v. inoculation of aqueous suspensions (Hollingdale & Lemcke, 1969).

Table 3. *Serological activity of* n-*butanol extracted lipid, lipid-free and recombined membranes of* Mycoplasma hominis *SC* 4

	Titre	
	Metabolic inhibition	Indirect haemagglutination*
Antiserum to:		
Purified membranes	160	5120
Lipid-free membranes	160	2560
Recombined membranes	320	5120
Antiserum to whole organisms absorbed with:		
Nil	1280	2560
Purified membranes	<10	10
Lipid	1280	1280
Lipid-free membranes	<10	<10
Recombined membranes	<10	<10

* Sera titrated against tanned erythrocytes sensitized with a sonicated suspension of SC4 at 0·125 mg. protein/ml.

Activities of antisera to membrane fractions separated by polyacrylamide gel electrophoresis

On polyacrylamide disk electrophoresis, membranes lysed with Triton X-100 migrated poorly and gave only a few ill-resolved bands, whereas those lysed with SDS gave 12–15 protein bands. Component 1 was found in sections containing protein bands which moved fastest in the gels whereas 2 and 3 were in sections closer to the origin. By inoculating rabbits with different gel sections, sera apparently monospecific to components 1 and 3 were obtained (sera A and B) but antisera to components 2*a* and 2*b* (C and D) also contained antibody to component 3 (Pl. 1*d* and *e*). Sera with antibody to components 1 or 3 both had MI titres of 40–80 whereas antiserum with antibodies to 2*a*, 2*b* and 3 had titres at least four-fold higher. Since components 2*a* and 2*b* but not 3 were inactivated at 60° C. for 30 min. (see below), antibody to component 3 was removed from one of these antisera (C) by absorption with membranes thus heated (serum Ca, Pl. 2*c*). MI and IHA titres were then reduced four- to eight-fold, to the same level as the 'monospecific' sera to components 1 or 3 (Table 4).

Properties of membrane components

Of the precipitating components identified by gel diffusion 2*a* and 2*b* were the most heat-labile; both were eliminated after heating at 60° C. for 30 min., whereas component 1 was inactivated at 80° C. and 3 was stable at 100° C. (Table 5). The proteolytic enzymes, pronase and trypsin, destroyed component 1 (Pl. 2*a*) and part of 2*b* (Pl. 2*b* and *c*). The latter is therefore complex. All components except 3 were destroyed by periodate and all except 1 by 0·1 N sodium hydroxide. Thus, 3 was the most stable. In preliminary experiments, pancreatic lipase and α-gluco-

Table 4. *Serological activities of antisera to membrane preparations of* Mycoplasma hominis *SC* 4 (*antiserum to whole organisms included for comparison*)

Rabbit inoculum		Protein inoculated (mg.)	Precipitating antibody to component	Antiserum titre: Metabolic inhibition	Antiserum titre: Indirect haemagglutination
Whole organisms		ND	1, 2*a*, 2*b*, 3	2560	2560
Purified membranes		3·5	1, 2*, 3	160	2560
Membranes lysed SDS		1·6	1, (2*a*), 2*b*, 3	160–320	320
Membrane proteins in polyacrylamide	A	ND	1	40–80	80
	B	ND	3	80	160
	C	ND	(2*a*), 2*b*, 3	160–320	160
	Ca+	ND	(2*a*), 2*b*	40	40
	D	ND	(2*a*), 2*b*, 3	320	2560
Urea extract		5·0	(1), 3	40	20
Phenolic extract		2·0	1, 3	320	1280

ND, not determined.
SDS, sodium dodecyl sulphate.
2*, 2*a* and 2*b* fused into one line.
(1), (2*a*), weak reactions in gel diffusion tests with detergent-lysed membranes.
Ca+, antiserum C absorbed with membrane suspension heated at 60° C., 30 min.

sidase had a similar range of activity to the proteolytic enzymes. However, both enzymes also degraded gelatin. This reaction was blocked by the addition of trypsin-inhibitor which also prevented the inactivation of the precipitating components. The lipase was still active against tributyrin in the presence of trypsin-inhibitor. Thus, both enzymes were contaminated with trypsin or a trypsin-like enzyme but neither alone had any effect on the precipitating antigens. Thin layer chromatograms of lipids extracted from membranes treated with phospholipases A or C were different from those of lipids from untreated membranes, suggesting some action on the membrane lipids. However, the precipitating components were unaffected by these enzymes. Similarly, β-glucosidase and α- and β-galactosidases were without effect.

The ability of treated membranes to absorb MI and IHA antibody decreased with the loss of precipitating components, although not strictly in parallel with the number of components destroyed (Table 5). All treatments diminished the capacity to absorb IHA antibody to a greater extent than the capacity to absorb MI antibody. In particular, heating membranes at 50° C. apparently left the precipitating antigens and the power to absorb MI antibody unaltered, but decreased IHA antibody absorption 16-fold.

DISCUSSION

The results of experiments with butanol-extracted lipid, lipid-free and recombined membranes suggest that MI and IHA antibodies are directed against membrane proteins rather than lipids or lipoproteins. This agrees with our previous results using whole organisms extracted with chloroform-methanol or aqueous acetone (Hollingdale & Lemcke, 1969). Although the chloroform-methanol and acetone extracts reacted weakly in gel diffusion and complement fixation, the butanol-extracted membrane lipid was completely inactive. This was probably

Table 5. *Serological activity of membranes of* Mycoplasma hominis *after various treatments*

Treatment	Precipitating components (gel diffusion) after treatment	Factor of decrease in antibody titre after absorption with treated membranes*	
		MI	IHA
Nil	1, 2*a*, 2*b*, 3	128–256	256–512
50°, 30 min.	1, 2*a*, 2*b*, 3	128	16–32
60°, 30 min.	1, 3	16	4–8
80°, 30 min.	3	2–4	0–2
100°, 30 min.	3	2–4	0–2
Sodium hydroxide	1	4–8	4
Periodate	3	16	2
Pronase	2*a*, (2*b*)†, 3	4	2
Trypsin	2*a*, (2*b*)†, 3	8	2

* Whole-organism antiserum with MI titre 1280–2560 and IHA titre 2560–5120 when held with buffer only.

† Part of component destroyed (Plate 2*a*–*c*).

because there was less protein in the solvent phase with butanol extraction than with the more drastic chloroform-methanol or acetone extractions. Kahane & Razin (1969) also found that MI antibody was directed against membrane proteins rather than lipids of *Mycoplasma laidlawii* and *M. gallisepticum*. These species, unlike *M. hominis*, ferment glucose. In contrast, membrane proteins of another glycolytic species, *M. pneumoniae*, elicit only low titres of MI antibody in rabbits and the specific glycolipid hapten must be re-aggregated with protein to obtain an adequate MI antibody response (Razin, Prescott & Chanock, 1970). Clearly, more species of mycoplasma need to be examined to elucidate the relative importance of membrane lipids and proteins in determining antigenicity and specificity.

The membrane proteins of *M. hominis*, like those of mammalian cells, were not easily rendered soluble. However, both urea and phenol extracted some of the antigens active in gel diffusion, MI and IHA, although the precipitating components which were later found to be the most labile (2*a* and 2*b*) were not recovered. The difference between the potencies of the antisera to the urea and phenol extracts was probably due largely to the differing systems of inoculation, since both extracts appeared to contain components 1 and 3 when tested against whole-organism antiserum.

The possibility of separating components from detergent-lysed membranes was explored because of the difficulty in obtaining membrane proteins in solution and the failure to separate the proteins in the urea extract by chromatography or disk electrophoresis. Although detergents denature proteins, this seemed to occur less than with the other reagents, since detergent-lysed membranes gave more complex precipitin patterns in gel diffusion tests. Moreover, membranes lysed with SDS still elicited the production of MI, IHA and precipitating antibodies, and detergent systems have been widely used in polyacrylamide disk electrophoresis of various proteins. In polyacrylamide gels, migration and separation of the proteins of membranes lysed with SDS was better than that of membranes lysed with Triton X-100. The non-ionic character of Triton X-100 may account for this and better migration might have been achieved in a buffer system at a different pH. Because of the small amounts separated in the gels when only 200 μg. of protein was applied to each, and the problem of elution, it was not feasible to assay the separated membrane components for MI and IHA activities. However, the attempt to raise antisera to the separated components was successful with respect to two of them and it was possible to make another serum specific to components 2*a* and 2*b* by absorption. Since all these antisera had MI and IHA activity it appears that several different antigens can give rise to MI or IHA antibodies. However, the precipitating antigens may not account for all the antigens involved; others not revealed by gel diffusion may be active. For example, antigens giving rise to IgM antibody, which does not react in precipitin tests, would not be detected by this system. This would apply particularly to antigen-antibody reactions in IHA, since IHA antibodies often belong to the IgM class of immunoglobulins (Pike, 1967). Indeed, the fact that antiserum to SDS-lysed membranes had an IHA titre eight-fold lower than that of antiserum to purified membrane, although it contained the same precipitins, suggests that some non-precipitating labile antigen(s) may have been

destroyed by the detergent. Another indication that antigen-antibody reactions other than those detected by gel diffusion occur in IHA, was the pronounced capacity of the urea and phenolic extracts for absorbing IHA antibody from whole organism antiserum. These extracts contained only components 1 and 3 and exhibited a limited capacity for absorbing MI antibody. Finally, the ability of membranes to absorb IHA antibody after heating at 50° C. was reduced even when the precipitating antigens were unaffected.

There were differences between the reactions of components 1, 2*a*, 2*b* and 3 to the various physical, chemical and enzymic treatments. Thus, 1 and 3 could be distinguished by their differing susceptibilities to heat, alkaline hydrolysis, periodate and proteolytic enzymes. The reactions of component 1, including inactivation by proteolytic enzymes, suggest that it is a protein. The resistance to the various treatments of the precipitability of component 3 militates against it being protein. However, it seems unlikely that this antigen is lipid in character, since it was neither extracted with butanol nor destroyed by pancreatic lipase or by phospholipases A or C under the conditions used. Both 2*a* and 2*b* were heat, alkali and periodate-labile. These components were also lost from membrane suspensions stored at 4° C. for 8 weeks, and even from frozen suspensions kept at −30° C. for 6–8 months. Component 2*a* differed from 2*b* in that it leached out into the suspending buffer when membrane suspensions were held at room temperature or 37° C. for several hours, whereas 2*b* remained associated with the particulate material. The lability of 2*a* and 2*b* suggest that they are proteins, although the precipitating activity of 2*a* was not destroyed by pronase or trypsin. The elimination of part of the precipitating activity of 2*b* by these enzymes shows that it is in fact a complex, part of which is protein. Thus, component 1 and part of the 2*b* complex could be identified as proteins. However, the periodate sensitivities of these components may indicate the presence of a carbohydrate determinant attached to the protein or a peptide with periodate-sensitive amino acids.

Although the determinant groups could not be precisely characterized by these experiments, their differing properties suggest that the components identified were chemically distinct from one another.

In an earlier paper we showed that the serological heterogeneity within the species *M. hominis* could be correlated with differences in the membrane antigens demonstrable by gel diffusion (Hollingdale & Lemcke, 1970). The question arises, therefore, whether strain SC4 is a typical representative of the species for antigenic analysis. However, our previous results showed that the three strains examined (including SC4), have some precipitating membrane antigens in common (Hollingdale & Lemcke, 1970).

Mycoplasma hominis is the first non-glycolytic, arginine-metabolizing mycoplasma whose membrane antigens have been examined. The membrane proteins are clearly important to the antigenicity of this organism, but more detailed biochemical work is required on this and other species of mycoplasma which do not have lipid or carbohydrate haptens like those in *M. pneumoniae* or *M. mycoides*, to characterize the determinant groups.

We thank Dr G. M. Gray and Mr A. Yates of the Biochemistry Department of this Institute for carrying out thin layer chromatography of the lipids of membranes treated with butanol or phospholipases and for assaying the activity of the glucosidases and galactosidases.

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EXPLANATION OF PLATES 1 AND 2

Gel diffusion reactions of membrane preparations of *Mycoplasma hominis* SC4.

PLATE 1

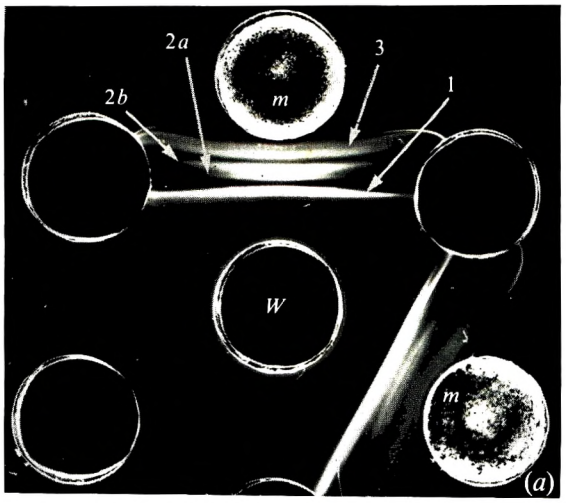
(*a*) Numbering of precipitating components (1, 2*a*, 2*b* and 3) in reaction between membranes lysed with Triton X-100 (*m*) and antiserum to whole organisms (*W*). Other wells contain medium, human serum and human γ-globulin all treated with Triton X-100, and Triton X-100 (2%, v/v) alone.

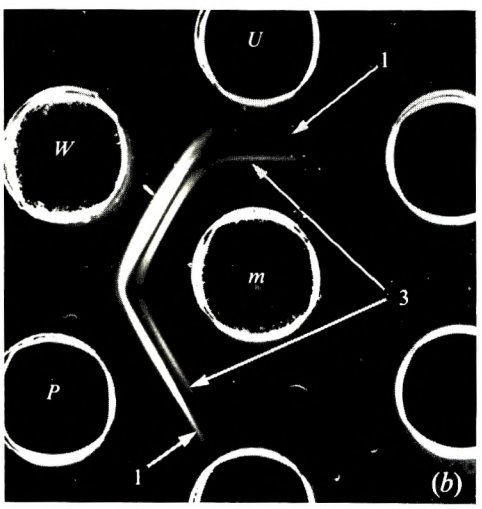
(*b*, *c*) Reactions between membranes lysed with Triton X-100 (*m*) and antisera to whole organisms (*W*), and to urea (*U*) and phenolic (*P*) extracts of membranes. Other wells contain medium.

(*d*, *e*) Reactions between membranes lysed with Triton X-100 (*m*) and antisera (A, B, C and D) against membrane fractions separated by polyacrylamide disk electrophoresis. Antiserum to whole organisms (*W*) for comparison. (Note components 2*b* and 3 are transposed with serum C.)

PLATE 2

(*a*–*c*) Reaction of pronase-treated membranes (*p*) with antiserum to whole organisms (*W*) and antisera (A, B, C and Ca) against membrane fractions separated by polyacrylamide disk electrophoresis. (Serum Ca is antiserum C absorbed with membranes heated at 60° C to remove antibody to component 3.) Untreated membranes (*u*) and buffer-treated membranes (*c*) as controls. All membrane preparations lysed with Triton X-100. Component 1 and part of 2*b* missing after pronase-treatment.


2a
3
2b
1
m
W
m
(a)


U
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W
m
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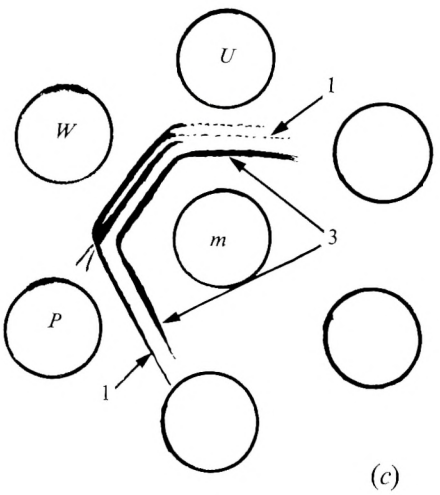
P
1
(b)


U
1
W
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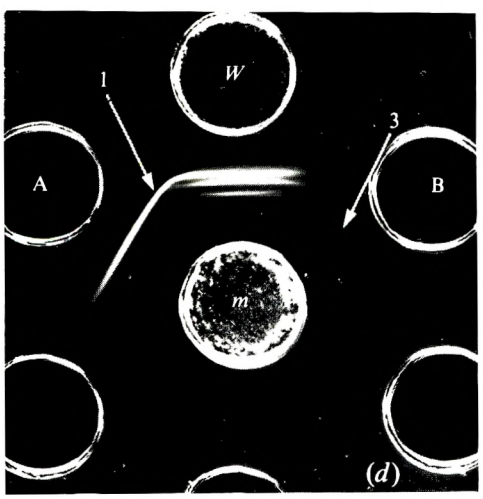
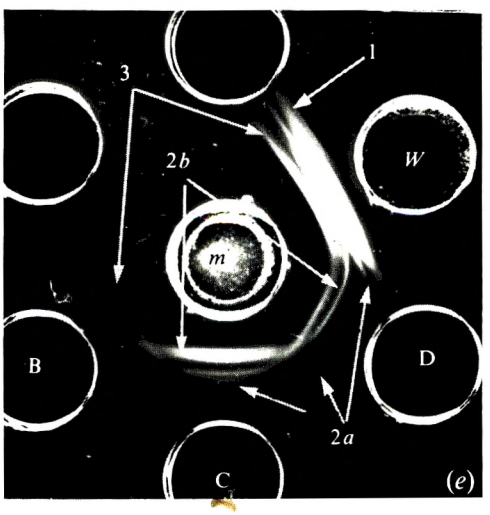
P
1
(c)


1
W
3
A
B
m
(d)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2b
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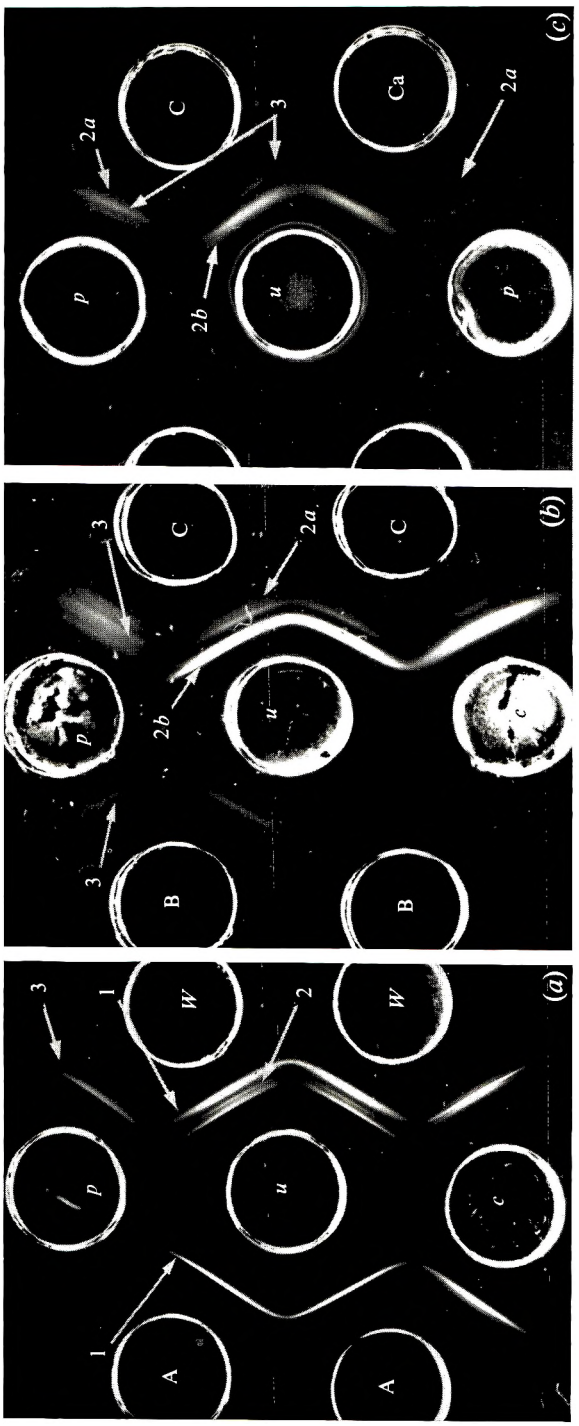
B
D
2a
C
(e)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Antibiotic resistance and transmissible R-factors in the intestinal coliform flora of healthy adults and children in an urban and a rural community

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SUMMARY

Faeces of healthy adults and of children under the age of 5, none of whom were attending hospital or receiving antibiotics, were examined for the presence of antibiotic resistant coliform bacilli.

A higher proportion of children (67 %) than of adults (46 %) carried resistant strains and this difference was observed in both the rural and urban groups.

Rural members of both age groups more often carried resistant organisms than urban members. Among rural adults, the incidence of drug-resistant strains was 63 % in those whose occupation involved close contact with farm animals, compared with 29 % in those with other occupations. The survey took place before the implementation of the Swann Report could have influenced the use of antibiotics in animal foodstuffs.

Transmissible R-factors were demonstrated in 61 % of the resistant strains. The incidence of transmissible resistance was similar among adults and children in town and country.

INTRODUCTION

Most studies of transmissible antibiotic resistance have been made with pathogenic Enterobacteriaceae or commensal coliform bacilli isolated from patients attending hospital. There have been fewer studies in normal populations. Smith & Halls (1966) found 20 drug-resistant *Escherichia coli* strains in the faeces of 15 of 24 healthy persons; 19 of these strains possessed R-factors. Datta (1969) found drug-resistant *E. coli* in the faeces of 52 of 100 adults admitted to hospital for non-urgent surgery; 60 % of the strains carried R factors. Moorhouse (1969) found drug-resistant faecal enterobacteria in 81 of 100 healthy Dublin infants below the age of 2; 71 of these were *E. coli* and 84 % of all the resistant strains possessed R-factors.

We report here a study of antibiotic-resistant coliform bacilli in the faeces of healthy adults and of young children in urban and rural populations. Our principal aim was to observe the effects of living in the country and of contact with farm animals on the prevalence of drug resistance, transmissible and non-transmissible, in normal human intestinal bacteria. The study took place before the results could have been much influenced by the recommendations of the Swann Report (1969) to restrict the use of antibiotics in animal foodstuffs.

MATERIALS AND METHODS

From June 1968 until July 1969, and again from May to July 1970, specimens of faeces were obtained from healthy children under the age of 5 years who lived in Bristol and attended infant welfare centres or were visited in their homes. Specimens were also obtained from adults some of whom were relatives of the children; but usually only one sample was obtained from one household. No one was receiving antibiotics or sulphonamides. Specimens from adults and children under 5 who lived in the country were obtained by visiting households in North Somerset, between July 1969 and April 1970. No one in the rural group had received antibiotics or sulphonamides for at least 6 months.

Faeces was plated on MacConkey agar. The investigation was confined to lactose-fermenting Gram-negative bacilli, hereafter referred to as coliform bacilli. Most strains were investigated by further tests (Cowan & Steel, 1965) which showed that 91 % of them were *E. coli* and the remainder *Klebsiella* spp. and *Citrobacter*.

The proportion of the faecal samples that contained large numbers of drug-resistant coliform bacilli was estimated during the first 12 months of the investigation by subculturing one lactose-fermenting colony chosen at random from MacConkey plates inoculated with faeces. (If colonial appearances suggested that there was more than one type present, one of each type was taken.) Sensitivity tests were made on nutrient agar containing 5 % haemolysed horse red cells, using Oxoid 'Multodisks' with ampicillin (25 μg.), streptomycin (25 μg.), tetracycline (50 μg.), kanamycin (30 μg.), nalidixic acid (30 μg.), chloramphenicol (50 μg.), nitrofurantoin (200 μg.) and sulphafurazole (500 μg.). A standard sensitive strain of *E. coli* was included with each batch of tests. All resistant strains were then tested for the presence of transmissible R-factors as described below.

Two methods were used to detect drug-resistant strains even when present in small numbers.

(i) Weighed samples of faeces were diluted 1/10 with sterile physiological saline and shaken for 1 min. in an M.S.E. homogenizer: 0·08 ml. of this suspension was spread evenly on a MacConkey plate and a 'Multodisk' placed on it. After incubation overnight, drug-resistant coliform colonies were selected, retested on lysed horse blood agar plates and examined for transmissible resistance.

(ii) Drops of 0·02 ml. of ten-fold dilutions of each faecal suspension were placed on 4 MacConkey agar plates; one plate was plain and the others contained 25 μg. per ml. of ampicillin, tetracycline or streptomycin respectively. Resistant coliform colonies were re-tested and examined for transmissibility.

Detection of transmissible drug resistance

Each resistant strain was tested by two methods:

(i) A mixture was prepared of 0·5 ml. of overnight broth culture of the resistant (potential donor) strain with 4·5 ml. of overnight broth culture of a non-lactose-fermenting nalidixic acid-resistant strain of *E. coli K. 12* and 5 ml. of sterile broth. After incubating this 'mating broth' at 37° C. for 20 hr., 0·02 ml. was spread on MacConkey agar containing nalidixic acid (50 μg./ml.) and the drug to which the donor was resistant (25 μg./ml.). A similar subculture was made after incubating the mating broth for a further 24 hr. Finally, the deposit obtained by centrifuging the mating broth was spread on a third MacConkey plate containing the drugs. Any non-lactose fermenting colonies growing on the plates were re-tested for resistance on haemolysed horse red cell agar.

(ii) R-factor transfer was studied quantitatively using a phosphatase-derepressed strain of *E. coli W. 3110* (Lee & Richmond, 1969).

RESULTS

In most specimens the predominant coliforms were sensitive to all the antibiotics (Table 1) but small numbers of resistant bacilli were present in more than half of the faecal samples. The more laborious method of plating several dilutions of faecal suspensions gave results only slightly higher than the simple procedure of placing a 'Multodisk' on a single plate inoculated with faeces. In determining the incidence of resistant strains in faeces, the results of both methods were combined together.

The incidence of antibiotic resistant strains was significantly higher in the faeces of children than of adults, in both rural and urban groups (Table 2). The effect of family occupation on the results can also be seen in Table 2. In the rural group 63 % of faeces from adult members of farming families contained resistant coliform bacilli compared with 29 % of adults in non-farming rural families, a significant difference. The difference between children of the farming and non-farming groups was slight, and not statistically significant. Similar differences were found between the proportions of individuals carrying strains resistant to each of the antibiotics separately (Table 3). Nearly all the resistant organisms were strains of *E. coli*.

Table 1. *Incidence of drug-resistant coliform bacilli in the faeces of healthy people (adults and children), tested by three methods (see text)*

Method	Total samples	Number with drug-resistant organisms
(i) Resistance of predominant organism	121	20 (17 %)
Detection of small numbers of resistant organisms		
(ii) Direct plating, with disk	264	142 (54 %)
(iii) Viable counts on antibiotic agar	264	154 (58 %)
(ii) and (iii) combined	264	156 (59 %)

R-factors. One hundred and ninety-eight drug-resistant strains were tested for transmissibility by both methods, with identical results. There was no statistically significant difference between any of the population groups; the proportion of resistance that was transmissible lay betweeen 53% and 72% (average 61%). The R-factors that were transferred are shown in Table 4.

DISCUSSION

Many different methods have been employed in surveys of antibiotic resistant bacteria and few workers have used identical techniques. The comparison of methods reported here indicates that simply placing a 'Multodisk' on a MacConkey plate inoculated heavily with faeces is remarkably efficient as a screening method. In experienced hands it can even provide reliable semi-quantitative data. The much more elaborate method of inoculating serial dilutions of homogenized faeces on a range of media incorporating different antibiotics does not appear worth while

Table 2. *Incidence of drug-resistant coliform bacilli in faeces of adults and children from an urban and a rural area. Influence of family occupation*

	Adults		Children		Total	
Source	Total samples	Samples with resistant organisms	Total samples	Samples with resistant organisms	Total samples	Samples with resistant organisms
Urban	97	41 (42%)	96	61 (64%)	193	102 (53%)
Rural						
Employed with livestock	43	27 (63%)	28	22 (79%)	71	49 (69%)
Other occupations	28	8 (29%)	17	11 (65%)	45	19 (42%)
Total rural	71	35 (49%)	45	33 (73%)	116	68 (59%)

Tests of significance for differences between groups:

Urban: Adults *vs.* children — $\chi^2 = 8{\cdot}75$, $0{\cdot}005 > P > 0{\cdot}001$

Rural: Adults *vs.* children — $\chi^2 = 6{\cdot}56$, $0{\cdot}025 > P > 0{\cdot}01$

Adults employed with livestock *vs.* other adults — $\chi^2 = 7{\cdot}94$, $0{\cdot}005 > P > 0{\cdot}001$

Table 3. *Resistance to individual antibiotics in coliform bacilli in faeces of healthy people*

	Adults with resistant organisms			Children with resistant organisms		
Antibiotic	Urban	Rural (farming*)	Rural (non-farming)	Urban	Rural (farming*)	Rural (non-farming)
Ampicillin	13 (13%)	18 (42%)	4 (14%)	35 (36%)	16 (57%)	8 (47%)
Streptomycin	17 (17%)	17 (39%)	6 (21%)	24 (25%)	13 (46%)	6 (35%)
Tetracycline	18 (19%)	20 (47%)	8 (29%)	23 (24%)	19 (68%)	10 (59%)
Sulphonamide	17 (17%)	14 (33%)	4 (14%)	16 (17%)	11 (39%)	3 (18%)
Chloramphenicol	4 (4%)	4 (9%)	0	5 (5%)	6 (21%)	1 (6%)
Total samples	97	43	28	96	28	17

* Employed with livestock.

for surveys of the kind described here, although it allows the determination of viable counts (and can provide suitable culture plates for replica plating techniques).

Considering this survey as a whole, resistant coliform bacilli were found in 49 % of faecal samples from normal adults, an almost identical figure to that reported by Datta (1969) in pre-admission specimens from patients at Hammersmith Hospital and similar also to the figure obtained by Datta *et al.* (1971) in pre-treatment faecal samples from adults with urinary infections. In faecal samples from children under 5, we found resistant coliforms in 67 %, similar to the results of Moorhouse (1969) from infants below the age of 2 years. The marked difference which we found between people of different ages within the same community and often within the same family is difficult to explain. One possible explanation might be a greater use of antibiotics among children than adults and so a short preliminary survey was made of the antibiotics prescribed during one week in September 1970 by 15 general practitioners from widely different parts of Bristol. The greatest use of antibiotics was among children of school age (antibiotics prescribed at a rate of 7·31 per 1000 patients on register), the least was in children of 0–4 years (4·71 per 1000) and adults received antibiotics at an intermediate rate (5·30 per 1000). This limited comparison suggests that the high incidence of resistant organisms in the bowel flora of young children may not be due to frequent antibiotic therapy.

The much higher incidence of resistant flora in the faeces of healthy rural families concerned with livestock, than in other rural inhabitants, was of particular interest,

Table 4. *Drug-resistance patterns in faecal coliform bacilli of healthy people, and the R-factors demonstrated in the resistant strain*

Antibiotic resistance patterns	Number of strains	R-factors (numbers transferred shown in parentheses)
A	43	A (8)
T	56	T (38)
S	4	0
A.T	24	A.T (21); T (1); A (1)
T.S	5	T.S (2); T (2)
T.Su	1	T (1)
S.Su	16	S.Su (11)
A.Su	1	0
A.Ne	1	A.Ne (1)
A.S.Su	12	A.S.Su (7)
A.C.T	1	A.C.T (1)
T.S.C	1	T.S.C (1)
T.S.Su	18	T.S.Su (13); T (1)
A.T.S	1	A.T.S (1)
A.T.S.Su	8	A.T.S.Su (4); A.T (1)
A.C.S.Su	1	A.C (1)
T.C.S.Su	3	T.C.S.Su (1); T.C (1)
A.T.S.Su.Ne	1	A.T.S.Su.Ne (1)
A.T.C.S.Su	1	A.T.C.S.Su (1)

Key

A	Ampicillin	Su	Sulphonamide
T	Tetracycline	Ne	Neomycin
S	Streptomycin	C	Chloramphenicol

although perhaps not surprising. The rural study was undertaken just after the publication of the Swann Report (1969) but it is almost certain that the restrictions in antibiotic use recommended by that report would have had little if any effect within the period of the survey. The occurrence of resistance to antibiotics, e.g. chloramphenicol, not used as foodstuff additives might be attributed to their use in treating sick animals. Whether the substitution of alternative growth promoters, as recommended in the report, will have the desired effect of reducing the antibiotic-resistant gut flora of farm animals or whether the substitutes will still be able to select bacteria carrying R-factors remains to be seen. We intend to make a further study of the intestinal flora of normal people living in the same regions, when the provisions of the Swann Report have been implemented.

We wish to acknowledge the co-operation of the health visitors and inspectors of the Public Health Departments in Bristol and Long Ashton who collected the samples for us, and in particular, Mr A. P. D. Nichols whose enthusiastic help was of great value.

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Bacteriuria in a Scottish island community. A comparison of chemical and cultural tests for bacteriuria applied in remote surroundings

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SUMMARY

Four hundred and thirty-eight overtly healthy adults on Tiree were screened for bacteriuria by dip-inoculum culture and a tetrazolium reductase test.

Dip-inoculum culture affords a simple and effective means of providing a service in quantitative urine bacteriology for communities remote from a laboratory.

The pattern of bacteriuria on Tiree is much the same as in other communities surveyed. Criteria are discussed for assessing the sensitivity of the tetrazolium test in terms of quantitative urine culture.

INTRODUCTION

In April 1969 a health screening team visited Tiree as part of a study of several distinctively different Scottish communities. Islanders attending the screening clinics were asked to bring with them a freshly voided sample of urine to be tested for protein and other abnormal constituents. This provided an opportunity to screen the population for bacteriuria and it was decided to compare the results of parallel tests by dip-inoculum culture, using the Mackey–Sandys spoon (Mackey & Sandys, 1965, 1966) with triphenyltetrazolium reduction (Simmons & Williams, 1962), using B.D.H. bacteriuria reagent.

METHODS

Dip-inoculum cultures were taken on plastic spoons (Medical Wire & Equipment Co., Potley, Corsham, Wilts.) charged with Oxoid brand CLED medium (Mackey and Sandys, 1966). This is an indicator-lactose medium with electrolyte content

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sufficiently low to prevent the spreading of *Proteus*. Cysteine is added to promote the growth of exacting coliforms. Sterile spoons were loaded with medium, placed in wide-mouthed one-ounce vials containing a moist pad of plastic foam and pasteurized in batches for 4 hr. at 70° C. in a thermostatic oven. At the screening clinics, cultures were prepared by momentarily dipping a culture-spoon in each urine sample. After dipping, the spoon was replaced at once in its vial to drain in the upright position onto the foam pad. The accumulated cultures were sent in three batches by air and rail to Bristol and thence by road to the laboratory at Bath. Each batch spent 4–5 days in transit at ambient temperatures prevailing in late April. After arrival at the laboratory the cultures were incubated overnight at 36° C. before being examined. Urine bacterial counts were estimated from the dip-spoon colony counts, using the established relationship that the urine count in organisms per ml. is 500 times the total surface viable count on the dip-spoon (Mackey & Sandys, 1965). Organisms were identified by their distinctive colonial appearances on CLED medium, supplemented when necessary by stained smears or further examination of subcultures. Drug sensitivity tests were done by a conventional disk-diffusion method.

Triphenyltetrazolium chloride (TTC) reductase tests were done on 2 ml. amounts of urine in tubes containing buffered TTC (British Drug Houses), incubated for 4 hr. in a thermostatic water bath at 37° C. A red precipitate of formazan indicates urine containing 100,000 organisms per ml. Tests were done by the island general practitioner in his surgery. Urine samples from the clinics were kept at 4° C. until tested.

RESULTS

Colony counts on dip-inoculum cultures are known to be unaffected by delays in transit of up to 48 hr. before incubation at 36° C. (Mackey & Sandys, 1965). In view of the extra delay between Tiree and Bath tests were made on the effect of storage for 5 days at 15° C. before incubating. Serial ten-fold dilutions were made in broth from cultures of selected pathogens. Replicate dip-inoculum cultures were made from each dilution over a range likely to include one producing between 10 and 50 colonies on each culture-spoon. A number of spoons were incubated at 36° C. immediately, while the remainder were held at 15° C. in a dark wine-cellar

Table 1. *Effect on D.I.C. spoon colony counts of storage at 15° C. before incubation overnight at 36° C.*

Organism	Mean dip-inoculum colony counts per spoon		
	Initial	After 48 hr. storage	After 5 days storage
E. coli	32	32	26
P. mirabilis	53	56	51
Klebsiella sp.	59	NT	54
Micrococcus sp.	14	NT	17

for 2–5 days before being incubated. Table 1 shows that 5 days storage had no significant effect on the colony count.

Each dip-inoculum culture was placed in one of the following five assessment categories according to the kinds of organism present and the estimated urine bacterial count.

1. Obscured by contamination (heavy confluent growth of non-pathogens).
2. No significant bacteriuria (less than 10,000 organisms/ml.).
3. Active infection indicated (more than 100,000 potential pathogens/ml.).
4. Contamination rather than infection (mixed pathogens/non-pathogens totalling more than 10,000/ml.).
5. Equivocal viable count (between 10,000 and 100,000 potential pathogens/ml.).

Table 2 shows the distribution of TTC test results in relation to assessment by dip-inoculum culture. Potential primary pathogens are regarded as comprising *Escherichia coli*, lactose non-fermenting (LNF) coliforms, *Klebsiella* sp., *Proteus* sp., *Pseudomonas aeruginosa*, *Staphyloccocus aureus*, and *Micrococcus* sp.

Table 3 summarizes the bacteriological findings from 22 specimens with more than 100,000 potential pathogens per ml. It should be noted that both strains of lactose non-fermenting coliform required cysteine.

Two to three months after the screening survey a second dip-inoculum urine culture was obtained from each of the 22 subjects whose first sample showed more than 100,000 potential pathogens per ml., taking particular care that freshly taken urine was examined. In 15 instances the results of the second culture were substantially the same as the first, but in seven the second examination did not fully confirm the first. Table 4 sets out the actual extent of the discrepancies noted. Subjects 1, 3 and 7 showed only small differences within the range of variation to be expected. Two subjects (2 and 6) showed a distinct fall in the number of organisms present, but the infecting organism remained unchanged. One subject showed no evidence of significant bacteriuria at the second test, while the seventh produced a lactose non-fermenting coliform quite distinct from the initial proteus

Table 2. *Tetrazolium reductase results in relation to dip-inoculum culture assessment*

Dip-inoculum culture assessment	Total	Tetrazolium reductase test	
		positive	negative
1. Obscured by contamination	23	18	5
2. No significant bacteriuria	374	8	366
3. Active infection indicated	22	18	4
4. Contamination rather than infection	14	7	7
5. Equivocal viable count	5	1	4

Table 3. *Organisms identified in urines containing more than 100 thousand potential pathogens per ml.*

Organism	Total cases
E. coli	17
Lactose non-fermenting coliform	2
Klebsiella sp.	2
Proteus sp.	1

infection, and present in rather smaller numbers. Repeat cultures were also taken from 31 subjects randomly chosen from the group with negative initial urines. In all cases the second culture proved unequivocally negative.

Since the TTC test is designed to detect urines containing more than 100,000 organisms per ml., Table 5 has been constructed for all samples on which the dip-inoculum culture enabled the count to be assessed, discarding the results from 23 samples so heavily contaminated that bacteriological appraisal was impossible.

DISCUSSION

Experience on Tiree bears out the practical value of dip-inoculum culture as the means of providing a service in quantitative urine bacteriology for communities remote in distance or travelling-time from a bacteriological laboratory. Dixon & Clarke (1968) and Dixon (1970) have drawn attention to the value of the technique in Canada. Dip-inoculum cultures prepared in the laboratory from broth with known counts of pathogens have shown little change in viable count despite standing for periods of up to 5 days at ambient temperature before being incubated at 36° C.

The comparatively small population on Tiree limits the conclusions that can be drawn concerning the prevalence of bacteriuria among the islanders, but the

Table 4. *Discrepancies noted in seven replicate dip-inoculum cultures*

Case	Sex	Age	First DIC assessment	Second DIC assessment
1	F	74	*E. coli* 10^5/ml.	*E. coli* 40,000/ml.
2	F	57	*E. coli* 10^5/ml.	*E. coli* 6000/ml.
3	F	59	LNF coliform 10^5/ml.	LNF coliform 30,000/ml.
4	F	72	*Proteus* sp. 10^5/ml.	LNF coliform 20,000/ml.
5	M	72	*E. coli* 10^5/ml.	No significant bacteriuria
6	M	18	*E. coli* 10^5/ml.	*E. coli* 6000/ml.
7	M	81	*E. coli* 10^5/ml.	*E. coli* 70,000/ml.

Table 5. *Sensitivity of tetrazolium test in relation to urine colony count and DIC assessment*

		TTC		
DIC assessment	Bacterial count	Total	Pos.	Neg.
Active infection indicated	More than 100,000 pathogens/ml.	22	18	4
Contamination rather than infection	More than 100,000 organisms/ml.	10	4	6
	Total	32	22	10
No significant bacteriuria	Less than 10,000 organisms/ml.	374	8	366
Contamination rather than infection	Less than 100,000 organisms/ml.	4	2	2
Equivocal viable count	Between 10,000 and 100,000 pathogen/ml.	5	1	4
	Total	383	11	372

general pattern resembles other reported surveys using dip-inoculum spoons (Grob, Manners & Dulake, 1970) and dip-slides (Heinonen *et al.* 1969). Asscher (1970) estimates that about 4% of women between 16 and 65 show significant bacteriuria. On Tiree there is the same marked preponderance of women over men and a general tendency for the condition to be commoner as age increases. Although the island population shows some excess of hypertension by comparison with the mainland communities (Hawthorne *et al.* 1969), the small numbers seen with bacteriuria permit no conclusion to be drawn on any possible association between hypertension and bacteriuria such as has been reported in larger studies (Asscher, 1970).

The organisms isolated in association with significant bacteriuria were representative potential urinary tract pathogens. It should be noted that both the strains of lactose non-fermenting coliform encountered were cysteine requiring, but produced colonies easily seen on CLED medium. This is specially formulated to include cysteine hydrochloride in view of reported association between urinary infection and cysteine-requiring coliforms, which produce only minute colonies on MacConkey agar and other traditional urine culture media (Gillespie, 1952). Discrepancies of the kind listed in Table 4 are to be expected when quantitative urine cultures are repeated. Even if attention is confined to subjects producing high counts of potential urinary pathogens, the possibility remains that these may occasionally appear as a consequence of accidental contamination rather than as evidence of active infection. It has also to be accepted that authentic active infection is not invariably attended on all occasions by high bacterial counts. Levy & Kass (1970) have proposed dealing with this source of uncertainty by examining two additional specimens following an initial positive sample. This

Table 6. *TTC sensitivity in detecting high pathogen counts*

(Specificity 0·82, 95% confidence limits lie between 0·60 and 0·95. Sensitivity 0·96. J = 0·6 (S.E. 0·10).)

	TTC test		
Dip-inoculum culture assessment	Positive	Negative	Total
Active infection indicated	18	4 (18%)	22
No significant bacteriuria Contamination rather than infection Equivocal viable count	15 (4%)	378	393

Table 7. *Detection of high bacterial counts by TTC test, without regard to status as pathogens*

(Sensitivity 0·69. 95% confidence limits 0·53 to 0·85. Specificity 0·97. J = 0·64 (S.E. 0·08).)

	TTC test		
Bacterial count	Positive	Negative	Total
More than 100,000 per ml.	22	10 (31%)	32
Less than 100,000 per ml.	11 (3%)	372	383

was not attempted on Tiree. Whilst implying that estimates of incidence are open to some error, this does not affect the validity of comparisons between the TTC test and dip-inoculum culture when applied to the same urine sample.

The results from dip-inoculum culture can be applied in two ways for the appraisal of the TTC test, assessing efficiency at detecting urines with high counts of potential pathogens or by the detection of any urine with a high count whether of pathogens or commensals. In Table 6 the data from Table 5 are arranged to provide a comparison in relation to the detection of urines with more than 100,000 potential pathogens per ml. Among 22 such urines the TTC test detected 18 (82%). This may be compared with values ranging from 79% to 91% reported by other observers examining various population groups. (Bulger & Kirby, 1963; Deutch & Jesperson, 1964; Kincaid-Smith *et al.*, 1964; Pinkerton, Gibson & Houston, 1964; Simmons & Williams, 1962; Williams & Simmons, 1963; Williams, Leigh, Rosser & Brumfitt, 1964; Wright, 1968). However, this estimate is based on a small number of positive samples and has a wide 95% confidence interval, lying between 60% and 95%. Youden's Index (J), which takes account jointly of sensitivity and specificity, is 0·60 (S.E. 0·10). At the upper level of sensitivity the test would be satisfactory for survey purposes, but there could be drawbacks in using for diagnostic purposes a test which may miss up to 40% of positive samples. Moreover, like all other chemical tests for bacteriuria the TTC test must work in terms of a single threshold level. To the possible loss of a number of cases with significant bacteriuria must be added the likely loss of information about urines with counts in the equivocal range between 10,000 and 100,000 potential pathogens per ml. In the Tiree series five urines gave counts of this order, of which the TTC test detected only one.

The TTC test is probably not uniformly responsive to all organisms. Norden & Kass (1968) observed that a test able to detect 100,000 *E. coli* per ml. may fail to indicate the presence of *Ps. aeruginosa* even when in 10-fold greater numbers. Williams *et al.* (1965) noted that their form of the test detected only 22% of significant bacteriuria due to Gram-positive cocci. The Tiree series included 10 urines with high counts due to non-pathogens (mainly diphtheroids and saprophytic Gram negative bacilli) of which only five gave positive TTC reactions. In view of this Table 7 was prepared to show the results from tests on all urines with high bacterial counts, whether due to pathogens or not. Appraisal in these terms leads to much the same conclusion as one based only on high pathogen counts. The sensitivity of the test is 69% (95% confidence limits 0·53 to 0·85) while Youden's Index is 0·64 (S.E. 0·08), values which do not differ significantly from those of Table 6. It appears that specificity of the TTC test is satisfactory, but that sensitivity is certainly too low for diagnostic purposes and possibly also for screening surveys.

No practical difficulty arose from using dip-inoculum culture as a screening procedure. All the facilities of a fully staffed bacteriological laboratory were successfully deployed on examining specimens collected from a community 500 miles and 5 days travel away. The method offers the substantial advantages that bacteriuria can be related precisely to the organisms responsible, and its

degree can be estimated in terms of the actual bacterial count rather than by reference to a fixed threshold. Information can be obtained about urines with counts in the equivocal range. Since the survey a diagnostic service has been provided for the island general practitioner on the basis of dip-inoculum urine culture. The time taken in the screening clinics by the dip-inoculum technique is a matter of only a few seconds per patient. No further testing need be done in the field and no additional equipment is required.

The whole of the laboratory examination is carried out in the centre to which the cultures are despatched. The overall results from the dip-inoculum survey in Tiree were in good agreement with other surveys for bacteriuria in overtly healthy communities.

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Enteritis due to *Salmonella panama* from infected ham

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SUMMARY

After the appearance of sporadic cases of enteritis due to *Salmonella panama*, baked ham from one supplier was implicated as the source of infection. No pathogenic organisms were isolated from the working surfaces of the factory involved or from samples of a day's bacon output, but *S. panama* was isolated from the factory sewers. Stool examinations of the 500 employees showed one man in the baked ham section to be excreting *S. panama*. He was removed from work and no further infections were reported from the district. The organism could no longer be found in the sewers.

Some weeks later, further infections were reported in the London and Southend areas, which could be traced to ham from the original source. Sewer swabs at the factory were again positive. A further examination of all the employees revealed three cases and 82 symptomless excretors. Eight of 192 family contacts were also found to be excretors. Trimethoprim-sulphamethoxazole appeared to have no effect on the carrier state.

Examination of the hams in cold store showed some to be infected with *S. panama*, and a number of these had been consumed in the canteen.

Subsequent examination of pigs at slaughter and pig food prepared locally failed to isolate *S. panama*. The source of infection at the factory is unknown.

THE OUTBREAK

Initial local infections

In April 1970 a village shopkeeper had a mild 2-day illness with diarrhoea and *Salmonella panama* was isolated from her stool. She blamed her illness on cooked ham she had eaten recently. Subsequently her parents, who lived with her, her husband and two children, became symptomless excretors and continued so for 6 months, despite a wide variety of treatment. The organism was also isolated from a road worker in the village, and thereafter his wife and three children became symptomless excretors for the next 4 months. Shortly after this, two children in an adjacent village developed gastro-enteritis and the organism was isolated from them. Their parents remained unaffected. The common factor among the victims was baked ham produced at a factory 3 miles away.

The original shop and adjoining living premises were clean and no pathogenic

organism was found in unwrapped foodstuffs or on working surfaces or refrigeration equipment. The shop was closed and the owners prohibited from handling unwrapped food.

Sewer swabs from the one other village store and from the two public houses failed to show salmonellas.

The bacon factory

This was the major industry in a rural area, employing about 500 people. On average 600 pigs were processed each day on 5 days a week and only rarely were animals held in lairage overnight. A complete range of raw and cooked pork was distributed in the south-east of the country, and the factory management claimed that their products were consumed by half a million people each day.

Investigations at the bacon factory

One week after the detection of the original case in the village, sewer swabs in the factory drains proved positive for *S. panama*. Swabs from the working surfaces in the ham and gammon steak areas of the factory were negative, as were samples from baked ham in local shops. A selection of a day's product of baked hams was examined, but no pathogens were found.

Because infection was obviously present in the factory, a stool sample from each employee was examined, and one man in the baked-ham area was found to be a symptomless excretor of *S. panama*. His main job was to operate the vacuum machine making gammon billets. He was removed from work and all subsequent stool examinations were negative. After three consecutive examinations he was allowed to return to non-meat-handling duties. His wife and two children remained negative throughout.

Further spread of the infections

This seemed to have removed the source of infection, as no further fresh cases were seen in the district for the next month. The sewer swabs in the factory also became negative. However, in the summer, sporadic cases began to appear in the London and Southend areas, in which baked ham from this factory had been eaten, and therefore might have been the cause. Exposed surfaces in the working area were again swabbed, including some areas remote from the suspected source of infection. *S. panama* was isolated from behind a baking oven, a meat packing table, a meat packing machine, and the conveyor belt in the gammon packing room. A factory order directed that working surfaces should be swabbed twice daily with a hypochlorite/detergent mixture, but there may have been lapses during heavy work periods. A square root sample from the week's product of baked ham in cold store was examined by slitting the plastic envelope and swabbing the surfaces, and six of 47 hams were found positive for *S. panama*. All hams were now withdrawn from retail outlets and destroyed. On Ministry advice, the hams in cold store were recooked in their covers at a temperature of 82° C to a depth of 2·5 cm and released for sale. At this time two workers reported with mild enteritis and *S. panama* was isolated from their stools. A further examination

of the employees showed 82 symptomless excretors, plus one further case with symptoms. Of 192 family contacts of positive individuals, eight were found to be excreting the organism.

The factory was now closed, new equipment installed, and work flow redesigned. After 2 weeks, employees with a negative stool examination were allowed to return to meat handling after a further negative examination. Excretors were allowed back after three consecutive negative stools, and thereafter the plant slowly returned to full production. No further cases could be traced to the product and no further isolations of *S. panama* were obtained from the factory or the personnel.

LABORATORY METHODS

Before this outbreak, a selective solid medium for the isolation of enteric pathogens – Hektoen enteric agar (King & Metzger, 1968*a*, *b*) – had been under investigation. This contains rather more peptone (1·2 %) than usual to offset the inhibitory concentration of bile salts (1·5 %) and sodium deoxycholate (0·2 %). The indicator system is bromothymol blue and Andrade's indicator, which produces a clear medium with a green background. The object was to identify salmonellas and shigellas on the same plate. Throughout the *S. panama* outbreak, a small outbreak of *Shigella sonnei* was running. This has not been reported. With pressure of work during the incident, technique was simplified to overnight incubation of faecal samples in single strength selenite at 37° C. followed by plating on Hektoen agar. After further overnight incubation, *S. panama* colonies showed up as clear to blue-green with a black centre; shigellas produced green colonies without blackening; and coliforms produced orange-coloured colonies. The medium was satisfactory as regards colour contrasts.

FACTORY METHODS

Baked ham and gammon steak

Boneless cuts of pork are soaked for 48 hr. in a solution of brine and saltpetre with added flavouring agents, hydrolysed vegetable protein, sugar and monosodium glutamate. The final concentration of salt in the cuts ranges between 3 and 3·5 %. They are then placed in metal moulds with spring lids and cooked for an average of 6 hr. in steam-heated ovens at 82° C. A sensor in the middle records a temperature of 70° C. and this must be reached before the hams are considered to be cooked. The moulds are then removed from the oven, the bases clamped down by manual pressure and passed through a cold shower. After cooling, the hams are removed from the moulds and wrapped. A small proportion are covered with breadcrumbs to suit regional tastes. The life of this product in the shop is about 1 week and they are normally held for 2 to 3 days in the factory cold store. For gammon steaks the pickled cuts are fed into a vacuum machine, which produces a cylindrical billet of uncooked meat wrapped in polythene. These are placed in an oven and the temperature raised to 37° C. over a period of 1 hr. They are held at this temperature for 1 hr. and finally steam-cooked until a sensor in the middle records a temperature of 53° C. This product requires further cooking before

consumption. The object of pickling is to flavour and preserve the product and it was thought that, as a result of this, the surface of the hams and gammons might acquire some bactericidal effect. Accordingly 1 in. squares of pickled gammon were inoculated with dilutions of an overnight broth culture of *S. panama* in quarter strength Ringer's solution to give inocula of about 5, 50, and 500 organisms. At weekly intervals a surface inoculated with each dilution was rubbed over a culture plate. At 4 weeks the organism could still be recovered from all dilutions. After 2 weeks storage at 4° C. ham and gammon had an unpleasant smell and appearance. During their edible life they obviously had no inhibitory effect on bacterial growth. *S. panama* could still be recovered from artificially infected ham stored at −20° C. for 1 year. Examination of the pickling fluid, cooling water, and breadcrumbs, yielded no enteric pathogens.

Salmonella panama

Incidence

In recent years the incidence of infections has been increasing, and Public Health Laboratory Service Reports (Vernon, 1966, 1967, 1969, 1970) and E. Vernon (personal communication) demonstrate the establishment of *S. panama* as an important human pathogen (Table 1).

Environmental contamination in outbreaks has been massive, with stools, window ledges, curtains, and personal items around the excretors heavily infected. In recent incidents the organism has been isolated from the blood and spinal fluid (*British Medical Journal*, 1971).

Properties

Organisms isolated from the original cases and from the bacon factory workers were dulcitol negative and sensitive to trimethoprim-sulphamethoxazole, ampicillin, tetracycline, chloramphenicol, colistin sulphate and neomycin but insensitive to streptomycin. At the end of the outbreak, the sensitivity pattern was unchanged.

Most Enterobacteriaciae can utilize nitrate anaerobically. In tinned corned beef the growth of *Salmonella typhi* is enhanced by up to 0·5 % sodium nitrate, but inhibited by 3 % sodium chloride (Meers & Goode, 1965*a*). These are the approximate concentrations in pickled meat and pork. Repeating the chequer board titration of Meers & Goode (1965*b*) we found that *S. panama* could grow at a salt concentration of 6 % but that growth was inhibited by the addition of 0·8 % sodium nitrate.

Table 1. *Incidents of* Salmonella panama *infection as reported by the Public Health Laboratory Service and associated laboratories*

Year	*S. panama*
1965	86
1966	95
1967	209
1968	379
1969	307

Incidence in pigs

S. panama is not a common pathogen of pigs in East Anglia. Since the beginning of the outbreak 500 caecal swabs from pigs killed in the factory and in the district have been examined for *S. panama* with negative results, although six other salmonella isolations were made: *S. typhimurium*, 3; *S. tennessee*, 1; *S. menston*, 1; and one unidentified serotype. Mesenteric gland swabs were examined with 154 of the caecal specimens and one was positive for *S. typhimurium*. The associated caecal swab was negative.

From 1958 to 1967 two porcine isolations were made from 19,371 incidents of animal infection at the Central Veterinary Laboratory, Weybridge (Sojka & Field, 1970), with three more in 1970 (W. J. Sojka, personal communication).

Incidence in animal feed

From 1969 until June 1971, 614 samples of raw material, mainly meat and bone meal from cattle food merchants in the area, were examined. There were no isolations of *S. panama*, but 141 samples (23 %) were positive for other serotypes, 26 in all.

No salmonellas were isolated from 124 samples of finished feed products. It would appear that steaming and subsequent pelleting sterilizes the product.

Treatment of carriers with trimethoprim-sulphamethoxazole

Apart from their usefulness in treatment of the typhoid group of fevers, antibiotics appear to have no beneficial effect on salmonella enteritis, and the carrier state may actually be prolonged by such treatment (Dixon, 1965; *British Medical Journal*, 1969). Some effective method of eliminating the organism from the bowel would be an advantage in the food industry, and trimethoprim-sulphamethoxazole seemed to offer some promise. It had proved effective in the treatment of typhoid fever (Kamat, 1970) and with a 4 to 5 weeks course Brodie, MacQueen & Livingstone (1970) reported that they eradicated the organism from two of five carriers of *S. typhi* and one of *S. saintpaul*.

Most of the bacon-factory workers were on the list of one group practice, and by arrangement one tablet of trimethoprim 80 mg. and sulphamethoxazole 400 mg. was given to 76 excretors four times a day for 10 days. Twenty-one excretors, acting as controls, received no treatment of any kind. After 1 month 28 of the treated and five of the untreated group were still excreting *S. panama*. After 2 months 11 (14 %) of the treated were still positive, but none of the controls

Table 2. *Length of infection in treated and untreated excretors*

Duration of infection	Treated patients	Untreated patients	Total
< 1 month	53 (70 %)	16 (76 %)	69
1–2 months	12	5	17
> 2 months	11	0	11
Total	76	21	97

(Table 2). Treatment appeared to prolong the carrier state, but the differences between the two groups are not statistically significant.

There were three instances of a skin eruption, two of the sufferers being withdrawn from the trial, and two of mild dyspepsia.

Many of the factory workers submitted weekly stools for examination for several months. In seven infected individuals a positive result was recorded after a mean of four negative examinations, and in one case, after eight negative examinations. These may have been due to a low level of residual bowel infection, but the possibility of re-infection from an unknown source remains. In most instances of salmonella infection, the symptoms are mild and intestinal carriage is self-limiting (Murdoch, 1971). Normal hygienic measures are usually sufficient to prevent family spread.

DISCUSSION

It is not known how infection entered the factory, although the original symptomless excretor may have been the culprit. After the outbreak caecal swabs from 500 pigs from the factory and surrounding slaughterhouses were examined and six salmonella isolations were obtained, none of them *S. panama*. About a quarter of the basic animal food ingredients examined locally contained salmonellas but steaming and pelleting seemed to eliminate this.

The original contamination of the hams may have been light. This is suggested because in the first 5 months of 1970 only 25 instances of *S. panama* infection were reported in the south-eastern region by the Public Health Laboratory Service and associated labaratories, 15 of them from this laboratory. Considering the popularity of cooked ham, this is a low score from a product which was widely distributed.

Although general hygienic measures at the factory appeared to be satisfactory, male cleaners tended to forget under surfaces of tables. However, it is likely that a clean working surface of low bacterial count was created twice a day on most working days.

Examination of stools from all employees, coupled with improved hygiene in the factory, should have detected excretors and dealt with any pockets of infection in the plant, but this did not prevent new infections. Apparently infected hams in the cold store were still being served in the canteen. The factory products were available to the workers at an economical price and were popular with their families, but considering that 192 were at risk and only 8 (4 %) were found to be excreting the organism, the contamination must have been light and intermittent.

The policy of the Public Health Laboratory Service regarding stool examinations of food handlers has been stated by the Director (Howie, 1970). As a purely routine measure in non-epidemic conditions no useful purpose is served.

The pickling process appeared to have no effect on the growth of *S. panama*, which would be expected from its salt tolerance, and a small inoculum on the ham was viable long after the end of its shop life. Treatment with trimethoprim-sulphamethoxazole appeared to have no effect on the carrier state.

Since the factory re-opened, the stools of all employees have been examined on

two occasions with negative results. Frequent examinations of sewers and working surfaces have been negative.

The source of the original infection remains a matter for conjecture.

We are indebted to Drs Russell, Hodgkin, Dean and Cockayne, who treated their patients with trimethoprim-sulphamethoxazole, and to numerous other practitioners who so kindly refrained from treating the controls, also to Miss Hilary Mogford who analysed the results.

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Salmonella aberdeen infection in cattle associated with human sewage

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SUMMARY

Salmonella aberdeen was established as the cause of illness in 30 out of a herd of 90 milking cows. The illness was only moderately severe, and all animals responded to treatment.

The source of infection was considered to be human sewage effluent overflowing onto grazing land. There was no report of human infection in the area from which the effluent came.

INTRODUCTION

There is an extensive literature on the transmission of salmonella infections from animal sources to man, and in the interests of public health, one would expect the subject to be well documented. The fact that transmission can occur from human sources to animals is also of importance, particularly from an epidemiological aspect, but receives scant attention in the literature. Only a few of the many reviews on salmonellosis, in particular one by Gibson (1965), discuss this possibility in any detail. As far as cattle are concerned, the source of infection is usually human sewage, after irrigation of pasture (Bederke & Lundt, 1954; Messerli, 1962); accidental contamination of drinking water (Hoflund, 1961); or accidental contamination of pasture (Piening, 1955). In some cases a method of spread cannot be definitely established, although a link exists between human and animal infection. Morten (1962) described an outbreak of *Salmonella typhimurium* infection in a dairy herd, where human infection with the same phage type had occurred in the same area, before the cattle outbreak. Lenk, Rasch & Bulling (1960), Hensel & Frerking (1964) and J. F. Harbourne (personal communication) all recorded cases of *Salmonella paratyphi* B infection in cattle, where there was strong evidence that the infection was derived from human sources.

This paper describes a herd outbreak of *Salmonella aberdeen* infection and its association with an overflow of human sewage onto pasture.

THE OUTBREAK

Description of herd and farm premises

The herd consisted of 90 dairy cows and approximately 70 young stock; dairy replacements were reared on the farm, but a few additional animals were purchased each year. In the winter the cows were housed in a covered yard and fed on hay and silage; during the rest of the year they were kept on a conventional

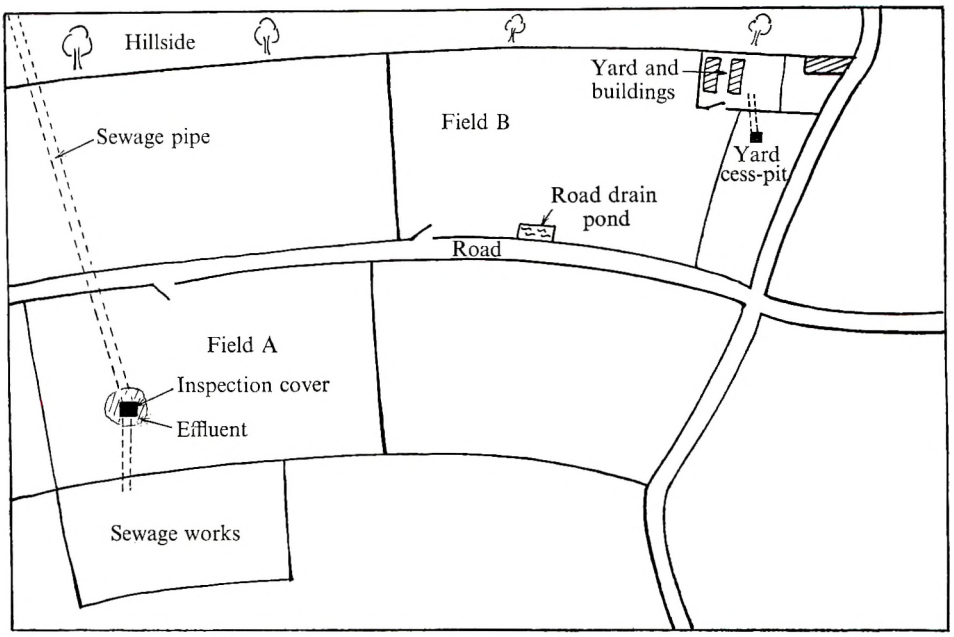

Fig. 1. Diagram of farm premises.

grazing system. Concentrates were fed all the year round, according to milk production.

The farm was situated on the slope of a hill and was made up largely of well-drained pasture land. The buildings, including the milking parlour, were in one corner of the farm, which was also separated into two parts by a road. Drinking water for the cows was from the main supply piped to troughs in the fields; the only other source of water accessible to the cows was a road drainage pond in a field adjoining the buildings. Immediately adjacent to the lower part of the farm was a sewage works, which dealt with material from a housing estate at the top of the hill; the main sewage pipe traversed the farm and was fitted with an inspection cover in one of the fields.

The essential details of the farm are shown in diagrammatic form in Fig. 1.

History

At the beginning of March 1970 the cows were turned out by day to graze a crop of rye in field A (Fig. 1). To reach this field they crossed field B, and walked along the road, returning by the same route for the afternoon milking. On at least one occasion during the month it was noted by the farmer that material had overflowed from the sewage pipe inspection cover into the rye, and this fact was reported to the local authority.

During April 1970 14 milking cows became ill, in batches of four or five at one time, over a 2-week period. The clinical signs included inappetance, moderate diarrhoea, pyrexia and marked drop in milk yield. Suspecting that this might be a salmonella infection, the owner's veterinary surgeon prescribed a 5-day course of

oral furazolidone (Neftin: Smith, Kline and French Laboratories Ltd) for each cow, at a dosage rate of 5 g. daily for 5 days; there was a good clinical response in every case. At this stage the possibility of infection arising from the overflowing sewage effluent was considered, and the cows were kept out of field A and allowed to graze only in field B (Fig. 1).

In late May 1970, whilst still grazing in field B, a further 16 cows became ill, nine being affected on one day. The signs included inappetance, slight diarrhoea, pyrexia, drop in milk yield and in approximately half the number, a severe mastitis. Once again oral furazolidone treatment was given and a good clinical response obtained. After this second period of illness the possibility of the road-drain pond having become a source of infection was considered, and it was fenced off. No further clinical cases occurred after this time.

METHOD OF INVESTIGATION

Inspection of the farm

A visit was made to the farm in early June, at the request of the farmer's veterinary surgeon. There were no seriously ill cows at this time, although the last group affected were still giving very little milk. Field A had been ploughed up and resown with barley, but the area of the inspection cover had been left undisturbed; it was covered to a depth of several inches with dried, matted effluent over an area approximately 6 ft. in diameter. The cover was found with difficulty, and removed; there was only a moderate flow of sewage material, but effluent similar to that above ground was heaped up on the benching. The road drain pond was fenced off; it contained only a small amount of water and was bordered on both sides by soft mud which showed many hoof imprints. The farm buildings were reasonably clean, but the adjoining yards were extremely dirty. Liquid material from this area drained into a cess-pit, which was overflowing.

The sewage effluent

Details of the effluent overflow in field A and the operations of the sewage works were obtained from the farmer; the information was later confirmed and amplified by the local medical officer of health. The works had been built during the war to deal with sewage from an American camp and closed shortly after the end of the war. It had been re-opened in January 1970 as a temporary measure to deal with sewage mainly from a new housing estate. Effluent was seen to overflow from the inspection cover on at least two occasions during March and April. Enquiries established that sewage material from cess-pit tankers was being pumped into the pipe at the top of the hill. The slope of the pipe and the fact that it changed direction at the inspection point, was sufficient to cause an overflow.

Public health aspects

At the end of June, investigations by the medical officer of health amongst the human population on the farm showed only the farmer to be a faecal excretor of *S. aberdeen*. The farmer's son and the dairyman had experienced bouts of diar-

rhoea at the time the cows were ill but both were negative at the time of sampling. No other cases of human *S. aberdeen* infection were reported from the rural district, including the area from which the cess-pit material came, during the whole of 1970.

Bacteriological examinations

Samples collected from the farm in June included rectal swabs from 80 cows; portions of the dried effluent; material from the sewage pipe benching, immediately beneath the inspection cover; mud and water samples from the road drainage pond; faeces from the farm-yards and material from the farm-yard cess-pit. At the end of August, approximately 4½ months after the first outbreak of infection, and 3 months after the first sampling, a second batch of rectal swabs was collected.

The rectal swabs and samples of effluent, pond mud, and faeces were incubated in selenite broth (Oxoid) at 37° C for 24 hr. and then plated out on deoxycholate citrate agar plates. After a further 24 hr. incubation non-lactose fermenting colonies were inoculated into Kohn Two-Tube medium (Oxoid) and incubated again. After 24 hr., cultures with the normal salmonella biochemical reactions were tested by means of salmonella agglutinating sera (Wellcome Reagents).

RESULTS OF BACTERIOLOGICAL EXAMINATIONS

A salmonella subsequently identified by the Salmonella Reference Labaratory, Colindale, and by the Central Veterinary Laboratory, Weybridge, as *S. aberdeen*, was isolated from 22 cows; from the sewage effluent; from material inside the sewage pipe; from two samples of pond mud; from the farm-yard cess-pit and from dung in the farm-yard.

DISCUSSION

Since *S. aberdeen* was isolated in June from 22 cows, all of which had been ill during April or May, and no other cause of disease was found, there can be little doubt that this organism was directly responsible for the illness.

The main methods of introduction of salmonellas into a dairy herd are by purchase of infected stock; by contamination of feeding stuffs, pasture or drinking water with faecal material of animal or human origin; by vermin or other animals; or in concentrated feeding stuffs.

In this case, no stock had been purchased since the previous year, and then only three animals; there was no history of flooding, or of contamination of pasture by any faecal material other than the sewage effluent; the cattle had no access to drinking water which was likely to be contaminated from outside sources. The farmer was found to be an excretor but his infection was almost certainly derived from the cows. Contamination of cattle food by rats was possible but unlikely, since regular control measures were used on the farm. Contamination of food or pasture by birds was a slight possibility; various salmonella serotypes have been found in birds, and there are records of association with disease in grazing animals (Gibson, 1965). Animal feeding stuffs are a recognized source of salmonellas, although it is established that the process of pelleting or cubing considerably

reduces the numbers present in the raw ingredients and that the finished product is likely to contain only small numbers of organisms (Report, 1959, 1961). On this farm, the concentrate food was a cubed product, supplied by a large national compounder; it was not examined for salmonella infection but seems unlikely to have produced such a large number of cases over a short period, on one farm.

There can be no doubt that of all the possible sources of infection, the sewage effluent was the most likely one. This hypothesis is supported by the illness of the cows that were in the field at the time the overflow was occurring; the illness of a number of cows at one time, suggesting a common source of infection; and the isolation of *S. aberdeen* from both effluent and sewage pipe. It can be postulated that the following chain of events occurred. The first group of cows became ill in field A, after eating rye contaminated by effluent. At this time they walked through field B on their way to and from milking, and contaminated the road drainage pond whilst drinking from it. When the cows were in field B all day the second group became infected through drinking from the pond, to which they had continuous access. As soon as access to the pond was prevented, no further illness occurred.

S. aberdeen was first typed in 1934, after it had been isolated from an infant suffering from acute enteritis (Smith, 1934). An extensive food poisoning outbreak in Britain in 1949 was recorded by Brockbank, Brown & Parker (1950) and sporadic cases of human infection were recorded by Taylor *et al.* (1965) and Vernon (1967).

There are few records of isolations from animals. Mallman, Ryff & Matthews (1942) and Edwards, Bruner & Moran (1948) in the USA mentioned its isolation from poultry; Taylor *et al.* (1965) recorded single isolations from the hippopotamus and an unspecified bovine in Africa and from the pig and sheep in India. An analysis of the salmonella incidents diagnosed by the Veterinary Investigation Centres of England and Wales and the Central Veterinary Laboratory, Weybridge, for the period 1958 to 1967 inclusive (Sojka & Field, 1970) makes no mention of *S. aberdeen*; in addition no incidents were recorded from these laboratories for 1968 or 1969 (W. J. Sojka, personal communication).

A number of isolations have been made from dried egg and egg products, (Newell, Hobbs & Wallace, 1955, Taylor *et al.* 1965) and occasional isolations from natural water and sewage (Taylor *et al.* 1965).

The lack of records of *S. aberdeen* infection may be due in part to its relatively low pathogenicity, or to its requirements for a special set of circumstances to produce disease. This may be an explanation, in part at least, for one of the puzzling features of this case, namely the presence in the sewage effluent of sufficient organisms to cause disease in a number of cows, when there is no evidence of previous human infection in the area from which the effluent came.

The overflow of effluent was directly due to improper use of a temporary but quite adequate sewage disposal system, and responsibility was accepted by the local authority. The farmer was fully compensated for loss in milk production which occurred whilst the cows were ill, a loss estimated at approximately 2000 gallons.

I wish to express my thanks to Dr V. N. Baker, Medical Officer of Health, for her invaluable assistance. I am also indebted to Dr B. Rowe, Salmonella Reference Laboratory, Colindale and to Dr G. Slavin, Bacteriology Department, Central Veterinary Laboratory, Weybridge for typing the salmonella cultures; to Mr W. J. Sojka for information and advice and to Messrs Cox & King, veterinary surgeons, for their co-operation.

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Salmonellas in pigs and animal feeding stuffs in England and Wales and in Denmark

BY A PUBLIC HEALTH LABORATORY SERVICE WORKING GROUP

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SUMMARY

A comparison has been made between the incidence of salmonellas in pigs and feeding stuffs in England and Wales and in Denmark. In Denmark there is veterinary legislation requiring the sterilization of imported and home produced feed ingredients of animal origin. There is no such legislation in England and Wales. In Denmark 0·3 % of resterilized imported meat and bone meal was contaminated with salmonellas. This compared with 23 % of meat and bone meal in England and Wales and 20–27 % of other ingredients of animal origin. In England and Wales salmonellas were isolated from 7 % of caecal samples and 6 % of lymph node samples, while in Denmark they were isolated from 3 % of caecal samples and 4 % of lymph node samples. In England and Wales 25 serotypes were found in both pigs and feeds and these included nearly all the most prevalent human pathogens. In Denmark four of the six serotypes in pigs had been found in resterilized feed. One notable difference between the two studies was the very wide range of serotypes found in pigs in England and Wales and the narrow range in Denmark. A second was that *Salmonella typhimurium* formed 15 % of all *Salmonella* strains isolated from pigs in England and Wales, and 60 % of those in Denmark.

It is concluded that sterilization of animal raw ingredients in Denmark has reduced pig infections with types other than *S. typhimurium* that are found in England and Wales, but not with *S. typhimurium*. It is possible that this is because *S. typhimurium* once introduced into pigs is able to establish itself more easily than other serotypes.

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INTRODUCTION

Since 1949, food poisoning reports by medical officers of health and bacteriologists in England and Wales have been collected and the information has been published yearly (Reports, 1950–64; Vernon, 1965–70). Over the past decade the annual number of food poisoning incidents due to salmonellas has fluctuated. Incidents caused by *S. typhimurium* fell sharply between 1959 and 1962. Since then there has been little change. Incidents due to other serotypes fell between 1959 and 1960 and remained fairly constant with minor fluctuations between 1960 and 1966. The 'other serotype' infections rose sharply between 1966 and 1968 when, for the first time, their numbers were greater than those caused by *S. typhimurium*.

A comparison of the number of incidents due to *S. typhimurium* and other serotypes in 1960 and 1968 is shown in Table 1. The relative importance of 'other serotype' incidents is greater now than in the past.

During the last 10 years, a number of serotypes have shown considerable fluctuations in incidence. Between 1959 and 1964, *S. heidelberg* and *S. brandenburg* were prevalent in man. After 1964 *S. panama* and *S. stanley* rose in human incidence and in 1967 the number of infections caused by *S. stanley* was second to those due to *S. typhimurium*. *S. panama* incidents have also continued to rise until in 1968 only *S. typhimurium* caused more infections in man. The prevalence of *S. panama* continues. *S.* 4, 12: d:–, an unnamed monophasic serotype isolated for the first time in England and Wales in 1968, was one of the most frequently found serotypes in 1969. In 1970, *S. agona* appeared and has become common. Pigs appear to be one of the main reservoirs of infection of these serotypes, but they have been found in poultry as well. Common sources of non-human isolations are pigs, pork products, drains of pig farms and bacon factories and pig slaughter areas in abattoirs. They are also isolated from animal feed ingredients.

Untreated animal feed in England and Wales is frequently contaminated with salmonellas. The part played by feeding stuffs in introducing new serotypes is probably significant, but their role in maintaining animal reservoirs of important human pathogens is difficult to assess. Pork and pork products are implicated each year in outbreaks of human salmonellosis. There is also some evidence from reports of salmonella isolations from pigs that they are a major source of salmonellosis in man, and it was thought that feed ingredients might be related to the maintenance of these animal reservoirs and also to the introduction of new serotypes. A com-

Table 1. *Comparisons of incidents due to* Salmonella typhimurium *and other salmonella serotypes in England and Wales in* 1960 *and* 1968

	1960*		1968†	
	No. of incidents	% of total incidents	No. of incidents	% of total incidents
S. typhimurium	2907	74	1654	43
Other serotypes	1047	26	2142	57

* Report (1961). † Vernon (1970).

parison of salmonellas isolated from pigs and their feed was arranged. Incidence and range of serotypes was investigated.

During the course of the study in England and Wales the Danish State Veterinary Serum Laboratory and the Institute of Microbiology and Hygiene of the Royal Veterinary and Agricultural University were approached and they agreed to carry out a similar project. There is strict veterinary legislation in Denmark requiring resterilization of imported feedstuffs of animal origin and sterilization of Danish animal products sent to rendering plants for destruction. A comparison between similar studies in the two countries was, therefore, considered useful in assessing the role of feeding stuffs in salmonella transmission.

MATERIALS

In England and Wales intestinal contents of pigs passing through abattoirs and raw ingredients of feeding stuffs distributed in the same area were examined. Bristol and Cardiff Public Health Laboratories had been monitoring feed ingredients for some years and it seemed reasonable to use the West Country of England and Cardiff in Wales as a suitable geographical unit for survey.

Samples of pig faeces were obtained and examined each week at six abattoirs by the Public Health Laboratories in Bath, Bristol, Cardiff, Exeter and Poole. The Bath laboratory examined pigs from two abattoirs, and the other laboratories from one each. Four of the five laboratories collected specimens by excising the caecum between two ligatures. Samples were placed in suitable containers for transport. In the remaining laboratory (Exeter) caecal contents were aspirated by a wide-bore Pasteur pipette.

Four laboratories also cultured mesenteric glands in addition to caecal faeces. In two, mesenteric glands and caecal samples were removed and examined from the same pigs.

Feeding-stuff ingredients were obtained at Avonmouth from a compounder and examined regularly at Bristol and Cardiff. These ingredients, which are listed in Table 4, include the principal ones of animal origin, that were likely to be salmonella contaminated, used in the manufacture of compounds or concentrates or purchased by farmers for mixing on farms. They include both imported and home produced material.

The only regulations pertaining to feed ingredients manufactured in the U.K. are that meat and bone meal should have certificates stating that the plant is capable of sterilizing this material.

In Denmark pig samples were obtained from two slaughterhouses. Faeces were removed from the caecum in the intestinal cleansing department into plastic beakers and portions were transferred at the abattoir to tetrathionate broth. Lymph nodes (ileo-caecal and part of mesenteric) were removed during meat inspection, cooled immediately to 0–5° C., and cultured the following day in tetrathionate broth. In some cases glands and faeces were collected from the same pig, but this was not a general procedure. These specimens were all examined at the Institute of Microbiology and Hygiene of the Royal Veterinary and Agricultural University.

Examination of feeding stuffs was carried out at the State Veterinary Serum Laboratory in Copenhagen.

Imports of meat and bone meal into Denmark were prohibited in 1933, but re-established after the war. Each shipment had to be accompanied by a document certifying that the consignment had been adequately heated (moist heat at 115–135° C. for at least 1 hr., or dry heat at 140° C. for at least three hours). Between 1949 and 1954, 1357 samples of this heat treated material were examined and 41 (3·6 %) contained salmonellas. Certification did not, therefore, exclude the occasional presence of food poisoning organisms. After March 1954 all imported meat and bone meal had to be resterilized at a Danish rendering plant in portions of not more than 25 tons.

Fish meal is not imported into Denmark. Danish fish meal is steam heated at 100° C and is reported to be free of salmonellas.

Home produced meat and bone meal has to be moist heat treated at 125° C. for 15 min. In addition there is a rigid separation in Danish rendering plants between the raw materials and the finished product. Plants are constructed so that there is no connexion to allow cross-contamination between the 'clean' and 'unclean' sections. Staff working in the two sections are kept apart by having separate eating, washing and toilet facilities.

There are no regulations requiring treatment of vegetable feed ingredients.

There were two separate series of examinations:

(1) Imported meat and bone meal sampled between 1949 and 1954, i.e. the period before compulsory resterilization, and from 1954 to 1970, i.e. the period after compulsory resterilization.

(2) The second series of samples were those actually fed to the pigs whose faeces and lymph glands were being examined. In the area studied, 90 % of all pig feeding stuffs came from two compounders. Both were willing to co-operate.

METHODS

In England and Wales, little alteration was made in techniques usually employed by the Public Health Laboratory Service for salmonella isolation. Selenite F broth, or tetrathionate broth, were used as fluid enrichment media. Incubation was carried out at 37 or 43° C. (Harvey & Thomson 1953) and subcultures were made at 24 hours to deoxycholate citrate agar, brilliant green MacConkey agar, or Wilson and Blair's bismuth sulphite agar. Personal preference naturally determined the method used. British technique was, therefore, less standard than Danish.

In Denmark, 10 g. of pig faeces were transferred to 100 ml. of tetrathionate broth to which 40 mg./l. of the sodium salt of novobiocin had been added. Incubation was carried out at 37° C. and subcultures made at 24 and 48 hr. to brilliant green lactose sucrose phenol red agar plates (B.L.S.F. agar). These were incubated at 37° C. and salmonella-like colonies were picked to triple sugar iron agar slants. Lymph node samples, immersed in boiling water for a few seconds, were cut into pieces by a scalpel and inoculated into tetrathionate broth without novobiocin.

Culture followed as described for faeces. No difficulties were encountered with swarming of colonies. In a few instances 0·02 % of Teepol was added to B.L.S.F. plates to prevent swarming. No improvement in results was obtained.

From each sample of feeding stuff, 10 portions of about 2 g. were cultured individually in approximately 30 ml. of ordinary nutrient broth. After 48 hr. incubation at 37° C., 2 ml. of each broth culture were transferred to about 30 ml. of tetrathionate. The enrichment media were incubated for 24 hr. at 37° C. and subcultured to brilliant green agar plates. After incubation at 37° C. salmonella-like colonies were picked for further identification and serotyping.

RESULTS

England and Wales

Details of samples and isolations were entered on two forms and sent to the Epidemiological Research Laboratory in London, where they were recorded and tabulated. A total of over 8000 pig samples and 1500 feeding stuff samples were examined, the majority in the period November 1968 to January 1970. Sampling has continued in some laboratories after this date.

Table 2 shows results of caecal sampling. Salmonellas were isolated from 7 % of 5637 pigs examined. In Bath, Cardiff, Exeter and Poole, where many specimens were cultured, the incidence varied from 2 to 14 %. In Bath, the laboratory recording the highest incidence, there was some degree of bias. More than a third of the samples came from a single farm on which salmonellas were found in feeding stuffs, pen samples and drain swabs.

Table 2 also records the isolations from mesenteric glands. The total incidence was similar to that found in faeces (6 % of 2483) and varied, in different areas, from 2 to 9 %. Two laboratories examined caecal contents and mesenteric glands from the same pigs during part of the investigation (Table 3). Isolation rates from the two sites were similar, but it was unusual for both to be found positive in the same animal. The proportion of pigs containing salmonellas approximately doubled when figures from glands and faeces were combined.

Table 4 provides information on feeding stuff ingredients examined and salmonella isolation rates. Salmonellas were isolated from 20 – 27 % of feed in-

Table 2. Salmonella *isolations from caecal samples and mesenteric glands of pigs, by five laboratories in England and Wales*

	Number of caecal samples		Number of gland samples	
Laboratory	Tested	Positive	Tested	Positive
Bath	628	88 (14)	—	—
Bristol	67	0	722	34 (5)
Cardiff	1281	115 (9)	281	13 (5)
Exeter	1940	45 (2)	610	14 (2)
Poole	1721	123 (7)	870	78 (9)
Totals	5637	371 (7)	2483	139 (6)

(The figures in parentheses indicate percentages.)

gredients of animal origin, with the exception of herring meal of which only 5 % of samples were contaminated. They were isolated from 1 % of sow nuts – a processed vegetable ingredient. It is noteworthy that 20 % of 264 samples of fish pellets contained salmonellas.

The range of serotypes found in the investigation was wide. Thirty-nine serotypes were found from 315 salmonella isolations from 5637 caecal samples, 23 serotypes from 104 salmonella isolations from 2483 gland samples and 52 serotypes from 313 isolations from 1772 samples of feed. Twenty-five serotypes were common to pigs and feed and these included several types prevalent in man. Table 5 lists serotypes found five times or more in each source, in order of frequency. In caecal samples, *S.* 4, 12: d:–, *S. typhimurium* and *S. dublin* were common, while *S. anatum* and *S. senftenberg* were often isolated from feed ingredients. Many other types were infrequently found. *S. dublin* was the commonest serotype found in mesenteric glands where it was found more frequently than in caecal faeces. In contrast, *S.* 4, 12: d:– had the maximum incidence in faeces and was relatively less common in glands.

Tables 6 and 7 contrast the incidence of serotypes common in pigs with those common in feeding stuffs. The tables are self-explanatory. Quantitative similarity of serotype incidence is not evident.

Denmark

Table 8 shows results of the Danish pig study, in which caecal samples and lymph node specimens were examined between May 1969 and July 1970 on 17 sampling days. Salmonellas were isolated from 8 (3 %) of caecal faeces and 15 (4 %)

Table 3. *Growth of* Salmonella *from caecal and gland samples taken from the same pigs, England and Wales*

		Pigs positive in			
Laboratory	Total pigs tested	Caecum and glands	Caecum only	Glands only	Total positive
Cardiff	276	3 (1)	13 (5)	10 (4)	26 (9)
Exeter	610	3 (0·5)	11 (2)	11 (2)	25 (4)

(Figures in parentheses are percentages.)

Table 4. *Growth of* Salmonella *from different ingredients of pig feed in England and Wales*

Raw material	No. samples	No. positive	Positive (%)
Feather meal	99	27	27
Raw materials [unspecified]	138	36	26
Meat and bone meal	704	163	23
Fish meal	31	7	23
Fish pellets	264	53	20
Herring meal	60	3	5
Sow nuts	162	1	1

of lymph nodes. *S. typhimurium* was the serotype most frequently found in lymph node samples.

In the period 1949–54, examinations of consignments of imported animal feed ingredients had shown that the accompanying heat treatment certifications were not proof of absence of salmonellas. Compulsory resterilization began in March 1954 and 10,782 samples representing about 270,000 tons of imported meat and bone meal were examined between 1954 and 1970. All this material had been reheated in Denmark after importation. Thirty-four samples (0·3 %) contained

Table 5. Salmonella *serotypes isolated from different sources, in England and Wales*

Caecum (5637 samples)		Gland (2483 samples)		Feeding stuff (1772 samples)	
Serotype	No. of isolations	Serotype	No. of isolations	Serotype	No. of isolations
S. 4, 12: d:–*	55	*S. dublin**	37	*S. anatum**	64
*S. typhimurium**	51	*S. typhimurium**	24	*S. senftenberg**	58
*S. dublin**	50	*S. eimsbuettel**	11	*S. livingstone**	29
S. chester	40	*S.* 4, 12: d:–*	11	*S. montevideo**	20
*S. panama**	23	*S. livingstone**	9	*S. oranienburg*	20
*S. fischerkietz**	23	*S. heidelberg**	7	*S. reading**	17
*S. livingstone**	20	*S. rostock*	6	*S. typhimurium**	15
*S. stanley**	12	*S. montevideo**	5	*S. tennessee**	15
*S. bredeney**	10			*S. eimsbuettel**	11
*S. indiana**	9			*S. infantis**	10
S. agama	9			*S.* 4, 12: d:–*	10
*S. heidelberg**	7			*S. cubana**	10
*S. anatum**	6			*S. ruiru*	8
*S. senftenberg**	5			*S. bredeney**	7
S. nagoya	5			*S. dublin**	7
*S. eimsbuettel**	5			*S. thompson**	6
*S. montevideo**	5				

* A serotype common to pig and feeding-stuff samples. Only serotypes that have been isolated five or more times are included in this table.

Table 6. *Isolation rates from all sources of some* Salmonella *serotypes which are common in pig samples (England and Wales)*

	Number of times specified *Salmonella* serotypes were grown from		
Serotype	Caecum	Glands	Feeding stuffs
All serotypes	371	139	367
S. 4, 12: d:–	55 (15)	11 (8)	10 (3)
S. typhimurium	51 (14)	24 (17)	18 (5)
S. dublin	50 (14)	37 (27)	7 (2)
S. chester	40 (11)	0	0
S. fischerkietz	23 (6)	2 (1)	2 (1)
S. eimsbuettel	5 (1)	11 (8)	10 (3)
S. livingstone	20 (5)	9 (6)	29 (8)

(Figures in parentheses are percentages.)

Table 7. *Isolation rates from caecal samples and glands of some* Salmonella *serotypes which are common in feeding stuffs (England and Wales)*

Serotype	Number of times specified *Salmonella* serotypes were grown from: Feeding stuffs	Caecum	Glands
All serotypes	367	371	139
S. senftenberg	60 (16)	5 (1)	3 (2)
S. anatum	64 (17)	6 (2)	4 (3)
S. livingstone	29 (8)	20 (5)	8 (6)
S. montevideo	22 (6)	5 (1)	5 (4)
S. oranienburg	20 (5)	0	0
S. reading	17 (5)	1 (1)	1 (1)

(Figures in parentheses are percentages.)

Table 8. Salmonella *isolations from pig samples taken from two abattoirs in Denmark*

Isolations from samples of: Caecal faeces		Lymph nodes	
Total samples	296	Total samples	359
Total positive	8 (3 %)	Total positive	15 (4·2 %)
S. typhimurium	2	*S. typhimurium*	12
S. montevideo	3	*S. montevideo*	1
S. senftenberg	1	*S. dublin*	1
S. oranienburg	1	*S. bispebjerg*	1
S. indiana	1		

Table 9. Salmonella *isolations from imported meat and bone meal in Denmark*

	No. of samples	No. positive
Before compulsory resterilization 1949–54	1,337	41 (3·6 %)
After compulsory resterilization 1954–70		
Post-sterilization samples	10,782	34 (0·3 %)
Pre-sterilization samples	278	47 (16·9 %)

Table 10. Salmonella *serotypes isolated more than five times from imported meat and bone meal in Denmark before sterilization*, 1949–70

	Period A	Period B		Period A	Period B
S. cubana	4	10	*S. give*	2	6
S. anatum	3	9	*S. bredeney*	3	4
S. typhimurium	6	6	*S. montevideo*	1	6
S. oranienburg	2	8	*S. senftenberg*	0	7
S. minnesota	2	7	*S. kentucky*	0	6

Period A, 1949–54 (Feb.). Period B, 1954 (Mar.)–1970.
The figures show the number of times each serotype was isolated in each period.

salmonellas. In the same period 278 such specimens were examined before resterilization. Forty-seven (17 %) were positive for salmonellas (Table 9).

Table 10 lists serotypes in frequency order isolated from imported meat and bone meal between 1949 and 1970. This table refers only to samples which had not undergone compulsory resterilization, while Table 11 shows serotypes isolated from resterilized imported feed ingredients sampled after 1954.

Table 12 records salmonella isolations from feed samples supplied by the two compounders involved in the Danish study. Two out of 206 samples were positive. One isolation was made from a pelleted mixture, the other from a meal. *S. typhimurium* was the serotype found. It was present in only one of 10 portions of a single sample (see section on Methods).

Comparison of results in England and Wales and in Denmark

From Tables 2, 5 and 8 the results of pig examinations in the two countries can be compared. The percentage isolation rate from caecal samples was 7 % in England and 3 % in Denmark, that from mesenteric gland samples 6 % in England and 4 % in Denmark. Forty-three serotypes were isolated in England and seven in Denmark. *S. typhimurium* accounted for 60 % of all isolations in Denmark, but only 15 % in England.

From Tables 4 and 9 a comparison may be made between the results of examination of imported meat and bone meal in Denmark and those of meat and bone meal examined in two British laboratories, Bristol and Cardiff. In England and Wales the present rate of contamination of 23 % compares well with that of Denmark (16·9 %) in the period after 1954 before resterilization was actually carried out. In England and Wales 40 different serotypes were isolated, compared with 32 in Denmark from all unsterilized material from 1949 to 1970.

Table 11. Salmonella *serotypes isolated from resterilized imported meat and bone meal in Denmark, after* 1954

Serotype	No. of times isolated	Serotype	No. of times isolated
S. senftenberg	7	*S. enteritidis*	1
S. montevideo	6	*S. meleagridis*	1
S. typhimurium	5	*S. minnesota*	1
S. cubana	5	*S. oranienburg*	1
S. alachua	3	*S. pullorum*	1
S. enteritidis-danysz	2	*S. tennessee*	1
S. anatum	1		

Table 12. Salmonella *isolations from feed from compounders supplying two pig abattoirs in Denmark*

Sample	No. of samples	No. positive	Serotype isolated
Feed mixture (pelleted)	88	1 (1 %)	*S. typhimurium*
Feed mixture (meal)	96	1 (1 %)	*S. typhimurium*
Meat and bone meal	11	0	—
Fish meal	11	0	—
Total	206	2 (1 %)	

DISCUSSION

In England and Wales 7 % of pig faeces and 6 % of mesenteric glands were positive for salmonellas. Percentages can be misleading unless it is realized that technique has a bearing on results. In Cardiff, in one series studied, inocula of 80 g. of faeces were compared with 0·5 g. Both inocula came from the same pig. The larger inoculum gave an incidence of 10 % of salmonellas, the smaller 5 %. The inoculum of glands used might also have to be taken into account in interpreting percentages. Obviously the figures for the group as a whole are merely averages, and technical variations and their possible influence cannot be evaluated.

When glands and faeces were examined from the same pig, it was rare for both sites to be found positive in the same animal. If isolations from both samples were combined the number of pigs harbouring salmonellas approximately doubled. The animals were symptomless as far as is known.

A recent study of salmonellosis in pigs in Holland identified a higher proportion of positive animals (20–50 %) than was found in the British investigation. Multiple faecal examinations were made during life and after slaughter in the Dutch series and this may well account for the difference (Kampelmacher, Guinée & Keulen, 1965).

In England previous pig investigations were carried out by Scott (1940) who examined mesenteric lymph nodes and spleens of slaughtered pigs at two bacon factories. Thirty-eight isolations were made from 1000 pigs (3·8 %) and eight serotypes were found. In a similar investigation in 1944–5 (Report 1947), 133 (2·5 %) salmonellas were isolated from 5285 pigs, which included 17 different serotypes. From the faeces of 600 healthy pigs, Smith & Buxton (1951) isolated four salmonellas (0·66 % positive) and only two serotypes. A working party of the Public Health Laboratory Service (Report, 1955) examined 5166 samples of lymph nodes and other tissues and an isolation rate of only 0·4 % was obtained with only three serotypes. Smith (1959) isolated 60 salmonellas from 500 pigs (12 %) which included 16 serotypes.

All these surveys have varied in size. They have been carried out in areas differing in size and locality and different sampling methods have been used. In the present survey the isolation rate in the different laboratories varied from 2 to 13 %. The isolation rate from one farm in which the feed was known to be contaminated was 18 %. It is thus clear that there are local factors operating and these may well vary from time to time so that it is difficult to make strict comparisons. However, it can be said that the present study has led to the isolation of many more serotypes in pigs (43) than have previously been isolated (17).

In 1964 Bevan Jones *et al.* showed that there was a relationship between the salmonella isolation rate in mesenteric glands and the length of stay of the pigs in the lairages. A 5–10 % isolation rate of two serotypes was obtained from pigs with a short stay in lairages (less than a week) and up to 90 % in pigs with a long stay (a week to a month). Slaughterhouse regulations of 1966 have forbidden the keeping of animals for longer than 72 hr. The pattern of pig salmonellas found in England and Wales in the present investigation suggested the source of infection. The same salmonella serotype was not usually isolated in succession during the

investigation, unless a number of pigs from the same farm were involved. This indicated that pigs were probably arriving at abattoirs already infected from farm or market and were not contracting salmonellas in the abattoir. Had the reverse been the case, animals from different sources would more likely have been infected with the same serotype on consecutive sampling days. Only one episode of this type occurred, when *S. chester* was isolated on 37 occasions in a single week.

Twenty-five serotypes in British pigs were also found in feed ingredients distributed in the same area. All types of ingredients sampled are fed to pigs. Meat and bone meal was the material most frequently sampled and 23 % of specimens contained salmonellas.

In 1968, over 90 % of meat and bone meal fed to United Kingdom livestock was home produced and nearly all serotypes common to pigs and feed were present in this material. This indicated the existence of a potential cycle of salmonella infection through abattoir and butcher waste products back to animals.

Approximately 75 % of fish meal and herring meal processed by large compounders, such as the firm involved in this study, is imported. Twenty per cent of the imported fish pellets examined from many batches were contaminated. Pelleting is known to minimize salmonella contamination if sufficient heat is employed. The pelleting in this instance was carried out at sea and may be a 'cold' process, i.e. heat below sterilization temperatures is used. Contamination of one batch of South African fish pellets may have occurred after manufacture. Salmonellas could have been introduced in transit to the United Kingdom in ships cleaned with sewage-polluted water. Alternatively, contamination could have occurred in Britain by contact with other contaminated material containing salmonellas, or from contaminated dust from plant environment. This is known to occur in other similar trades.

Approximately half the feather meal used by compounders is home produced and the other half imported.

Sow nuts contain vegetable ingredients (barley, wheat), vitamins and essential trace elements that are fed to pigs.

Of the serotypes isolated from feeding stuffs, *S. typhimurium* was found 15 times and is sixth in order of frequency. It is often suggested that, as this serotype is seldom present in animal feed, transmission by this route is unlikely. Few would deny, however, that *S. typhimurium* is frequently found in animal tissues (Table 5) and that these are sent to rendering plants for conversion into animal feed stuffs. Isolation of *S. typhimurium* from feed ingredients may be difficult. For example, isolation of *S. typhimurium* from bone meal is very much dependent on the technique used (Harvey & Price, 1970).

The 25 salmonellas common to pigs and feed in Britain included nine of the ten most frequently found human pathogens encountered in 1969. The nine serotypes were – *S. typhimurium*, *S. enteritidis*, *S. panama*, *S. heidelberg*, *S. stanley*, *S.* 4, 12:d:–, *S. indiana*, *S. infantis* and *S. dublin*. The first and last serotypes are more commonly isolated from cattle than from other animal sources. *S. virchow* was frequently found in man in 1969, but this serotype is now associated with

poultry and our failure to find it in pigs is not surprising. Most of the other serotypes have been commonly found in poultry as well.

The incidence of salmonella types found both in feed and in pigs shows little quantitative correspondence (Tables 6 and 7). We do not think that this invalidates the potential association. Certain serotypes may survive and multiply differently in different environments and those which readily multiply in the pig intestine may not do so in pig and other animal remains from which animal raw ingredients for feeds are derived. Enterobacteriaceae show differences in this respect and, in examination of bones from India and Pakistan, salmonellas have frequently been isolated from samples *not* containing *E. coli*. This has also been experienced with other dried food materials (Harvey and Price; unpublished).

In the Danish investigation, salmonellas were found in pigs' faeces and mesenteric glands despite sterilization of ingredients of animal origin, but the incidence was less than that in England and Wales – 3 % in faeces and 4 % in glands.

The most striking difference between Denmark and Britain is the narrow range of pig serotypes found in the former country and the wide range in the latter. *S. typhimurium* isolations were 60 % of all isolations in Denmark and only 15 in England. This may be of great significance. During the period 1960–8 *S. typhimurium* accounted for 80 % of all isolations in humans in Denmark. During the same period in England *S. typhimurium* accounted for 60 % of human incidents in man, but this has been getting progressively less and in 1968 *S. typhimurium* accounted for only 43 % of incidents while in the same year it accounted for 64 % of isolations in Denmark.

It is certainly evident that exotic species are not frequently found in pigs or humans in Denmark and this must be due to the heat processing of animal feed. It is likely that the cycle of *S. typhimurium*, the commonest type in pigs and humans in Denmark and other countries, is independent of feed transmission. However, it is still isolated from feeds so that it is also possible that an occasional introduction of *S. typhimurium* in the feed is sufficient to set up a persistent infection in pigs. Intensification of pig farming and animal husbandry in Denmark is similar to that in England except that with the colder climate in Denmark more pen houses are enclosed with greater control of temperature, humidity, lighting and ventilation. It is possible that in these conditions *S. typhimurium* once introduced is able to survive and multiply and so give rise to persistent cross infection.

The salmonella incidence in treated Danish meat and bone meal was 0·3 % as compared with 23 % in the United Kingdom. The finding of salmonellas in treated Danish feed suggests either insufficient heat treatment, or contamination of the treated product by contact with contaminated material, notably vegetable feed materials, which are not sterilized. In Britain it is known that feed pelleting is a rather critical process and it is possible for some failure to occur. Our experience also leads us to suppose that cross contamination from other contaminated materials is a potential danger.

As regards the role of imported feeds in Denmark, Müller (1952, 1957) showed that 'foreign' salmonella species had increased in incidence in animals and were

also found in man. These exotic serotypes were at that time associated with imported meat and bone meal. Since introduction of compulsory feed sterilization, the situation has changed and unusual species are seldom found in animals, but this does not mean that feed is no longer of any importance in Denmark. Vegetable feed ingredients are not sterilized and are known to be occasionally contaminated. In 1958 salmonellas were found in seven of 72 vegetable feed samples examined. It is also noteworthy that of the 7 serotypes that occurred in the Danish pigs, four have been found in resterilized imported meat and bone meal and three of these – *S. typhimurium*, *S. montevideo* and *S. senftenberg* – are the commonest from this source. *S. typhimurium*, the commonest type in the pigs, was found in two feed samples from the compounders supplying the feed to the pigs. The serotypes found in the pigs are also reported to be the types that have been found in dry rendering plants and feed-stuff factories in the warm, humid summer months.

Calculations made from the incidence of salmonellosis in Danish pigs suggested that five to ten salmonella carriers might pass through the abattoir in an hour. From swabs taken in the slaughter area no evidence of salmonella contamination of the slaughter line was obtained. Numerous examinations of meat products have also been made by laboratories authorized to perform bacterial meat inspection all with negative results. It is felt that improvements in abattoir hygiene that have taken place in Denmark in recent years prevent carcass contamination in spite of the demonstrated carriage of salmonellas by pigs.

In England and Wales, on the other hand, pork sausages and domestic pork products have been found in 1970 to contain *S. typhimurium*, *S. panama*, *S. stanley*, *S. indiana*, *S.* 4, 12:d:– and *S. enteritidis*. There is little doubt, therefore, that in this country pathogens find their way onto surfaces of carcasses and into the meat prepared from infected pigs.

We thank the following Health Departments for providing the basis for the pig observations by very patiently collecting the samples at abattoirs: Bristol, Exeter, Highworth Rural District and Wimborne and Cranborne Rural District.

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Salmonellas on pig farms and in abattoirs

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SUMMARY

Salmonella infection on two pig farms and its relation to infection in pigs at slaughter was studied. On the first farm feed ingredients were mixed on the farm, and these included fish meal which was found to be contaminated with salmonellas. The feed was pumped to pigs in liquid form. There was a high salmonella isolation rate at slaughter when the contaminated fish meal was fed in liquid feed, but it was significantly lower when no fish meal was fed to the pigs examined at slaughter. In some instances the same serotypes were found in fish meal and pig excreta on the farm and in caecal contents of the pigs at slaughter. No serotype was repeatedly isolated from any source and it appeared that the serotypes were not able to establish themselves in the pigs. It is concluded that infection found at slaughter originated on the farm where fish meal introduced and maintained infection. There was an opportunity for salmonellas to have multiplied in the liquid feed for several hours each day.

On a second farm environmental conditions were similar, but feed was given in the form of ready-made pellets and nuts. Salmonellas were not isolated from the feed. At slaughter there was a significantly lower isolation rate than on the first farm.

INTRODUCTION

The investigation of salmonellas in pigs and feeding stuffs in the West Country of England established that pigs at abattoirs commonly harboured salmonella serotypes pathogenic to man. Many of these serotypes were also present in feed ingredients before compounding. This suggested that contaminated feeding stuffs

could be a source of infection in pigs. It was realized that the compounded feeding stuffs actually consumed by pigs might not be so heavily contaminated, as the compounding in factories would dilute the contamination. In addition, certain farmers obtained their own ingredients and mixed them on the farm. We decided to examine, where possible, feeding stuffs consumed by pigs on farms and to investigate pig faeces on the farms and at slaughter.

It was hoped that this study would yield information on the role of feed in introducing salmonellas into pigs, and clarify problems of cross-infection and environmental contamination. We also wished to obtain information on changes of serotypes in the pigs corresponding to changes in the salmonellas found in the feeds.

FARM STUDIES

Farm A

During the course of an abattoir study, Bath Public Health Laboratory identified a farm on which pigs were infected with salmonellas. A detailed investigation of this farm was made over a period of one year; feed and pig faeces were sampled on the farm and caecal samples of animals from this farm were taken at the abattoir.

The farm was a semi-intensive fattening unit. It contained about 2000 pigs, up to 30 being housed in each pen. Pigs were taken from a number of breeding farms at 10 weeks and 60 lb. weight and fattened for 16 weeks to 200 lb. weight. There was no history of any clinical illness in the pigs.

The feed was mixed from purchased ingredients in a tank of approximately 6000 gallons capacity. Liquid feed was pumped through pipes to troughs in the pens which were above ground level. Drinking water from the mains was added to the troughs. Pipes were flushed with cold mains water once daily. The standard of hygiene was good; floors of pens were concrete and were hosed down daily.

Ingredients mixed by the farmer for the feed were millers' offal (flour), barley, a cereal mix, a vitamin supplement, fish meal and skimmed milk.

The majority of feed samples examined were fish meal as it was the ingredient most probably contaminated, but all other ingredients were also examined. Regular samples of pen faeces were obtained and some farm drain swabs were examined. Caecal samples of pigs from the farm were taken concurrently at the abattoir, but it was not always possible to examine the same pigs from which pen samples had been collected on the farm. Transport from the farm to the abattoir took three hours and pigs were slaughtered the following day.

Farm B

Farm B was investigated by the Dorchester Public Health Laboratory because of its different feeding practice to that of Farm A.

This farm was also a semi-intensive fattening farm obtaining pigs as 10-week weaners from several different sources. They were fattened for 12–14 weeks to a weight of 140–160 lb. There were just over 400 pigs which were housed under cover in pens each containing about 25 pigs. Pens had concrete floors and gal-

vanized dividing walls and were cleaned out each day. The animals fed themselves *ad lib.* from dome-shaped hoppers up to a weight of 100 lb. Above 100 lb. each pig was fed half a pound per day and the feed was placed on wooden sleeping boards in the pens. The feed was in the form of pellets and nuts and supplied ready-made by the manufacturers.

During the period of study droppings indicated that rats were present on the farm. There were 50 ewes on the farm which were kept separate from the pigs. There was no clinical illness in the animals or the farm workers.

Farm samples of feed were taken and caecal faeces and mesenteric glands from pigs at the abattoir. The abattoir was approximately an hour's journey away and the pigs were killed almost immediately after arrival. Sampling was carried out for a period of 3 months.

MATERIALS

Farm

Feed samples were collected into plastic bags, and pen faecal samples were taken with wooden spatulas from the floors and placed into waxed cartons (Mono), which were packed in large plastic bags. Sewer swabs were placed in the main pig drain and removed the same day or left for a few days before removal. They were sent to the laboratory in sterile glass jars.

Abattoir

Caecal samples were removed from the pig at the abattoir by excising the caecum between two ligatures, placing it in a suitable container and sending it to the laboratory for examination.

Mesenteric glands were removed by dissecting out the gland and enucleating it from surrounding fat.

Methods

Selenite F broth, or tetrathionate broth, was used as enrichment medium. Incubation was carried out at 37 or 43° C. (Harvey & Thomson, 1953) and subcultures were made at 24 hr. to deoxycholate citrate sucrose agar, brilliant green MacConkey agar (Oxoid) and Wilson and Blair's bismuth sulphite agar (Oxoid). Plates were incubated at 37° C. for 24–48 hr. and suspicious colonies picked for further examination.

Quarter strength Ringer's solution and nutrient broth were used for pre-enrichment for feed samples and incubated at 37° C. Double strength selenite broth was then added to the enrichment cultures.

RESULTS

Farm A

Table 1 shows results of all sampling on Farm A and the abattoir over the 1-year period. During this time, abattoir caecal samples were collected on 24 occasions and feed and pen faecal samples from the farm on 14 occasions. Fifteen of 18 fish meal samples contained salmonellas. Thirteen of these 18 samples were imported

meal and all were positive. One of three British fish meals was positive and one out of two meals of unknown origin. Seven samples of feed ingredients other than fish meal were examined and only one (a sample of millers' offal) contained salmonellas. Eleven of 136 pen faecal samples and two of seven drain swabs taken on the farm were positive. At slaughter 58 (18 %) of 316 caecal samples contained salmonellas. Twelve serotypes were found in fish meal and thirteen in all feed samples. Seven were found in pig excreta (faeces and drain swabs) and seven in caecal samples at slaughter.

Table 2 shows the timing of isolation of serotypes from different sources. Dates on which positive samples were obtained are shown and number of isolations of each serotype. One or two fish meal samples were taken on each occasion, about a dozen pen faecal samples, one farm drain swab and 6 to 12 caecal samples.

In general, no serotype was isolated consistently or consecutively from a source for any length of time, although for 1 month *Salmonella panama* was found on three sampling occasions from caecal contents at slaughter. The monophasic serotype *S.* 4, 12:d:– was most frequently isolated from all sources. *S. stanley*,

Table 1. *Salmonella isolation rates and serotypes according to source of sample*

	Source	Total samples	No. positive	Serotype	
Farm A	Fish meal	18	15 (83)	*S.* 4, 12:d:–*	4
				*S. stanley**	2
				*S. anatum**	2
				*S. fischerkietz**	2
				S. cubana	2
				S. derby	1
				S. infantis	1
				S. oranienburg	1
				S. agama	1
				S. potsdam	1
				S. orion	1
				S. senftenberg	1
	Other feeds	7	1 (14)	*S. kralingen*	1
	Pen faeces	136	11 (8)	*S. thompson*	4
				*S. typhimurium**	2
				S. 4, 12:d:–*	2
				S. hvittingfoss	1
				*S. anatum**	1
				*S. fischerkietz**	1
	Drain swabs	7	2 (29)	*S. stanley**	1
				S. 4, 12:d:–*	1
Abattoir	Caecal	316	58 (18)	*S. panama*	21
				S. 4, 12:d:–*	13
				*S. stanley**	9
				S. livingstone	9
				*S. typhimurium**	5
				S. heidelberg	2
				*S. fischerkietz**	1

Figures in parentheses are percentages.
* Serotypes common to more than one source.

S. 4, 12:d:– and *S. fischerkietz* were found in fish meal, pig excreta on the farm and also in faeces at slaughter, and *S. anatum* in fish meal and in pigs on the farm. Sometimes the same serotype was present in more than one source within a short time. *S. stanley* was found in two samples of fish meal, taken within 4 days (April 1969) and in pig sewage on the farm on the same day as the second sample; 2 weeks later *S. stanley* was isolated from caecal contents of five pigs at slaughter (May 1969). *S. anatum* was found in two samples of fish meal in a period of 6 weeks (June and July). Six weeks later (September) it was present in a pen faecal sample. *S.* 4, 12:d:– was found in two samples of fish meal and in two samples of farm faeces on the same day (November); 2 weeks afterwards it occurred again in fish meal and 10 days later in pig caecal samples. *S. fischerkietz* was found in a sample of fish meal (November) and 10 days subsequently in caecal faeces at the abattoir.

Table 2. *Farm A. Time relationship of salmonella serotypes isolated on farm and at abattoir*

Date	Fish meal		Pig excreta (farm)		Caecal samples (abattoir)	
10. iv. 69	—		—		*S. livingstone*	9
20. iv. 69	—		—		*S. typhimurium*	2
					S. 4, 12:d:–	1
25. iv. 69	*S. stanley*	1	*S.* 4, 12:d:–	1	—	
29. iv. 69	*S. stanley*	1	*S. stanley*	1	—	
14. v. 69	—		—		*S. stanley*	5
21. v. 69	*S. derby*	1	—		*S. heidelberg*	2
12. vi. 69	—		—		*S.* 4, 12:d:–	5
18. vi. 69	*S. anatum*	1	—		—	
	S. potsdam	1	—		—	
30. vii. 69	*S. anatum*	1	*S. hvittingfoss*	1	—	
	S. fischerkietz	1	—		—	
16. ix. 69	*S. cubana*	1	*S. anatum*	1	—	
	S. 4, 12:d:–	1	—		—	
8. x. 69	—		*S. fischerkietz*	1	—	
23. x. 69	—		—		*S. stanley*	4
5. xi. 69	*S.* 4, 12:d:–	2	*S.* 4, 12:d:–	2	—	
	S. oranienburg	1	—		—	
18. xi. 69	*S. agama*	1	—		—	
	S. infantis	1	—		—	
	S. fischerkietz	1	—		—	
	S. 4, 12:d:–	1	—		—	
28. xi. 69	—		—		*S.* 4, 12:d:–	6
	—		—		*S. fischerkietz*	1
3. xii. 69	—		—		*S.* 4, 12:d:–	1
4. iii. 70	*S. cubana*	1	*S. typhimurium*	2	—	
	S. orion	1	—		—	
11. iii. 70	—		—		*S. panama*	3
16. iii. 70	*S. senftenberg*	1	—		—	
8. iv. 70	—		—		*S. panama*	6
14. iv. 70	—		—		*S. panama*	12
21. iv. 70	—		*S. thompson*	4	—	
29. iv. 70	—		—		*S. typhimurium*	3

Change of feed

Sampling was carried out for just over 1 year, from April 1969 to May 1970. As a result of changes in feeding practice, this period could be divided into three separate phases (Table 3). The changes involved the animal protein constituent, the other feed ingredients remained constant. The first period ran from early April 1969 to the end of November 1969, during which time pigs were fed on imported fish meal. This was added to the feed mixture in the proportion of approximately 100 lb. per ton. At the end of November supplies of imported fish meal were stopped and it was replaced by skimmed milk as the main animal protein ingredient. This second phase stretched from the end of November 1969 to the end of January 1970 during which time no foreign fish meal was added to the feed mixture, although a reduced ration of British white fish meal (14 lb. per ton of total feed) was fed in a dry form by hand only to small pigs (10–14 weeks old). The third period stretched from the end of January 1970 to the end of May 1970, when a similarly reduced ration of fish meal was added to the liquid feed mixture and fed to all pigs. During February, the source of the fish meal was unknown, but from February onwards only British white fish meal was fed.

Table 3 compares the salmonella isolations from all sources during these three periods. During the first period (April to November 1969) all 13 samples of fish meal were positive and 5 of 101 pen faecal samples grew salmonellas. At the abattoir 148 caecal samples were taken, of which 33 (22 %) were positive.

In the second period (December 1969 to January 1970) no pen faecal samples or feed samples were taken and only one of 60 caecal samples at slaughter was positive.

In the third period (February to May 1970), two of five fish-meal samples and six of 35 pen faecal samples were positive. Twenty-four (22 %) of 108 caecal samples were positive.

It was only in the second period (December and January) that contaminated fish meal was not fed in liquid form to any pigs and not fed at all to older pigs (14–26 weeks old) awaiting slaughter. In the first and third periods contaminated fish

Table 3. *Farm A. Comparisons of salmonella isolations during three phases of feeding*

	April–November 1969	December 1969–January 1970	February–May 1970
Fish meal fed	100 lb. imported fish meal per ton in liquid mash to all pigs	14 lb. British fish meal per ton fed dry to small pigs	14 lb. British fish meal in liquid mash to all pigs
Fish meal samples	13/13 positive	Not examined	2/5 positive
Pen faecal samples	5/101 positive	Not examined	6/35 positive
Caecal samples	33/148 positive	1/60 positive	24/108 positive

meal was fed through the liquid feed system to all pigs. The caecal isolation rate (1/60) for the second period was highly significantly lower than that of the first (33/148) and the third (24/108) period ($\chi^2 = 14{\cdot}2$, significant at the 0·1 % level).

Farm B

Table 4 shows results of sampling on Farm B. Samples of feed on the farm were taken on five occasions between January and March 1970. Eight samples of pig concentrate pellets and one sample of pig nuts were all negative for salmonellas. Among other ingredients the pig pellets contained meat and bone meal, meat meal and dried milk.

At the abattoir caecal contents and mesenteric glands were examined from 29 pigs on four sampling occasions during February and March 1970. There was one positive caecal sample (3 %) and one positive mesenteric gland (3 %). Positive samples came from different pigs in a batch of eight. *S. typhimurium* was cultured from both.

DISCUSSION

It has been possible through these studies to investigate the relation of farm infection to abattoir infection and the relative importance of feed and farm environment in the ultimate infection of pigs at slaughter. Environmental factors on the two farms were similar, but feeding practice different. On Farm A, replacement of one feed by another assisted in evaluating the part played by feed.

The findings on Farm A suggest that contamination of pigs at slaughter originated on the farm and not at the abattoir. The high isolation rate and the serotypes isolated at the abattoir from pigs from this farm did not occur in pigs from other sources passing through this abattoir which was one of six investigated in the West Country pig and feeding-stuff study (PHLS Working Group, Skovgaard & Nielsen, 1972). If abattoirs had been the main source of infection, similar isolation rates and the same serotypes would have been obtained from all pigs passing through the premises. Bevan Jones, Farkas, Ghosh & Hobbs (1964) showed that there was abattoir contamination of two serotypes (*S. brandenburg* and *S. typhimurium*) in lairages in which pigs were kept for periods of over a month and this led to a high isolation rate of these two serotypes of up to 90 % from mesenteric glands of these pigs at slaughter. Slaughterhouse regulations of 1966 have forbidden the keeping of animals for longer than 72 hr. Pigs from Farm A were slaughtered within 12–18 hr.

Table 4. *Salmonella isolations on Farm B*

	Source	Total samples	No. positive	Serotypes
Farm	Pig concentrate pellets	8	0	—
	Pig nuts	1	0	—
Abattoir	Caecal contents	29	1 (3)	*S. typhimurium*
	Mesenteric glands	29	1 (3)	*S. typhimurium*

Figures in parentheses are percentages.

Serotypes were not consistently isolated from fish meal, pen or caecal samples. The longest period that a serotype was isolated was just over a month in the case of *S. panama* from caecal samples. This is interesting as *S. panama*, a serotype of increasing human importance, has been most commonly isolated from pigs and pork products. It may be considered to have a relative host adaptation to pigs. It was preceded by and replaced by different serotypes. It thus appeared that no serotype was able to become established in the pigs. There was no opportunity for long-term excretors to give rise to persistent infection as there was a continuous throughput of pigs and young pigs did not mix with older pigs. Serotypes could only have become established by a lasting contamination of the environment which did not occur.

Occasionally certain serotypes were found in the feed, the pig excreta on the farm and the intestinal contents at slaughter. In some instances there was a close time relationship between these isolations. On four of the eight occasions on which salmonellas were found in pen faeces there was a preceding or concurrent isolation of the same serotype in the fish meal.

Some of the serotypes were found in pig excreta on the farm and not in the feed. Smith (1960) also isolated some serotypes in the faeces of pigs fed contaminated feed from which the serotypes had not been isolated. He considered that these types had been present in the feed, but stated that there were technical difficulties in isolating individual serotypes from a number of other serotypes in materials such as fish and bone meal.

Other serotypes were found in the fish meal, but not in the pig excreta or in the caecal contents. This confirms observations by Smith (1960). There is evidence that the pig acts as a biological filter and that different serotypes have different infective properties for the pig. Newell *et al.* (1959) reported that, from the same contaminated food, pigs became infected with *S. orion* whilst poultry became infected with *S. infantis*.

Salmonellas virtually ceased to be isolated from intestinal contents when no fish meal was fed to adult pigs and that being fed to smaller pigs was in dry as opposed to liquid form.

Results on Farm A suggest that fish meal in the feed constantly introduced salmonella infection and that environmental contamination alone did not maintain this infection indefinitely. This confirms work done by Smith (1960) who showed experimentally that once feeding of salmonella-contaminated food to pigs stopped, excretion of salmonellas by the pigs could no longer be detected. His examinations were made from 6 to 20 days after ceasing to feed contaminated materials. In a study carried out in an intensive pig-breeding farm (Ghosh, 1972), four serotypes persisted over a 2-year period. Boars and sows were retained for long periods of over 1 year and they were moved around to different houses, thus permitting dissemination and perpetuation of infection. There were opportunities for persistent cross-infection which did not exist on the fattening farm.

It was reasonable to compare the results on Farm A with those on Farm B. Although Farm A had five times as many pigs as Farm B, the number of pigs to each pen was the same and the pens and standards of hygiene were similar. The

essential difference was the type of feed and method of feeding. On Farm B the pigs were fed on ready-made processed pellets and nuts and of nine samples examined not one was positive for salmonellas. At slaughter, caecal and mesenteric gland samples were examined from the same pigs. One out of 29 caecal samples (3 %) was positive and this was significantly different from the 18 % incidence in caecal samples from Farm A ($\chi^2 = 4{\cdot}2$, significant at the 5 % level).

The implication of the results on Farm A is important as over 50 % of pig-fattening farms in the United Kingdom mix their own feed. Most farms are not in a position to test raw materials, as large manufacturers do, and contaminated materials rejected by the big compounders find their way to farms. Few farmers have facilities for pelleting and the majority of mixed foods are in the form of meals. On the farm investigated, the mixed meal was fed in a liquid form. In a sample of 864 pig farms taken by the Meat and Livestock Commission, pipeline feeding with liquid feed was used in 10·6 % of farms (Meat and Livestock Commission, unpublished data).

Linton, Jennett & Heard (1970) investigated the question of salmonella multiplication in liquid feed. They showed that whilst approximately one salmonella was originally present per 1 ml. of liquid feed, as many as 200 salmonellas per ml. could be demonstrated after holding the feed at 20° C. for 24 hr. and 200,000 per ml. after 48 hr. at 20 and 28° C. This emphasized the fact that liquid feed held for a single day or residual feed left in feeding troughs and pipe-lines between feeds, could act as media for salmonella multiplication. They further showed that salmonella excretion was only transient following the feeding of freshly prepared mash; after feeding this mash on 2 consecutive days excretion lasted 24 hr. only. In contrast after a single feed of mash held for 24 hr. at 20° C. excretion of salmonellas could be demonstrated for at least 37 days.

On Farm A the liquid feed was made up each day using mains water and pumped into the troughs twice a day where it was usually consumed in $\frac{1}{2}$–$1\frac{1}{2}$ hr. The tank, which was under cover, was emptied once a day so that feed remained in the tank between feeds for about 7 hr. each day. The temperature of the feed would vary with the atmospheric temperature. It is known that salmonellas in foods can multiply at temperatures ranging from 7 to 45° C. (Angelotti, Foter & Lewis, 1961). There was thus the opportunity for salmonellas to multiply in the tank before the second feeding, and multiplication could also have taken place if there had been any residual feed in the troughs, pipe or tank.

The highly contaminated fish meal was foreign. Subsequent chemical analysis of a sample of this material showed that there were other ingredients present, including feather meal. About 75 % of fish meal fed to livestock in the U.K. is imported and such material is a likely source of exotic serotypes introduced into Britain.

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An epidemiological study of the incidence of salmonellas in pigs

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SUMMARY

The incidence of salmonellas in pigs was studied in five farms and a bacon factory.

Persistence and spread of salmonella excretion in pigs in a breeding establishment is described. Salmonella excretor boars and sows were responsible for the spread and perpetuation of infection in the farm. The possibility of spreading salmonella infection between farms through the distribution of excretor pigs was studied. Infection persisted and was related to the initial state of excretion of the pigs while at the farm of origin.

The importance of feeding stuffs as a source of salmonella infection in pigs is discussed. Specially prepared heat treated pellets fed to the pigs prevented the introduction of salmonellas.

INTRODUCTION

The wide distribution of salmonellas in nature and their importance as pathogens in animals and men are well established facts. The role of animals as a source of these organisms is recognized (Hobbs, 1961; van Oye, 1964; Greatorex, 1966). Pigs are considered to be an important reservoir (Buxton, 1957). Pig meat and meat products have often been found associated with food poisoning incidents in man (Bevan Jones, Farkas, Ghosh & Hobbs, 1964).

Salmonellosis occurs either as overt clinical infection or as symptomless excretion in man and animals. *Salmonella choleraesuis* and *S. typhimurium* are mainly responsible for clinical disease in pigs but many other serotypes are excreted in the faeces without symptoms, although localization often occurs in lymph nodes of young animals. It has been recognized that symptomless excretion is of potential public health importance although its incidence may not be known by veterinarians.

Earlier surveys included examination of pooled mesenteric lymph nodes from normal pigs at abattoirs (Hormaeche & Salsamendi, 1936, 1939; Scott, 1940; Medical Research Council, 1947). Smith & Buxton (1951) stressed the risk of contamination of carcasses and abattoir premises in a study of faecal samples from pigs at slaughter. Many workers have searched for the source of infection in pigs. Galton, Smith, McElrath & Hardy (1954) demonstrated the importance of abattoir lairages in cross-infection. Later work by McDonagh & Smith (1958) and Bevan Jones *et al.* (1964) showed the relation between build up of infection and duration of stay of pigs in lairages. Shotts, Martin & Galton (1962) demonstrated a consider-

able increase of excretion of salmonellas in pigs during transportation and subsequent holding at abattoir lairages until slaughter.

Newell and his colleagues (1959) noted examination of caecal contents from normal pigs at abattoirs gave higher isolation rates of salmonellas than caecal swabs. These workers traced the source of infection back to the farm of origin of infected pigs and were able to isolate the same serotypes from pigs and feeding meals.

Others have also investigated the source of infection of pigs on farms (Galton *et al.* 1954; Leistner, Johantges, Deibel & Niven, 1961). Intensive farming methods involve large numbers of susceptible animals being reared together on feed concentrate. A considerable proportion of this is derived from animal sources. In Holland, Edel & Kampelmacher (1970) isolated salmonellas from 30 % of healthy pigs at slaughterhouses and in Bulgaria 7–31 % of pigs were found to be symptomless excretors (Dimitrov *et al.* 1970).

In a study of pigs, mainly conducted in south-west England (PHLS Working Group, Skovgaard & Nielsen, 1972), 5637 samples of caecal faeces and 2483 of mesenteric lymph nodes were examined. Faecal incidence of salmonellas ranged from 2 to 13 % with an average of 6 %. Of the mesenteric glands, 5 % were positive for salmonellas and when both were examined the proportion of infected pigs was twice that obtained by examination of one site only.

The work presented here is concerned with some of the factors associated with the epidemiology of salmonellosis in pigs, including persistence of symptomless excretion, dissemination and sources of infection within the farm, and potential risk of spread between farms through distribution of excretor animals. Investigations were conducted at five farms and one bacon factory.

THE FARMS

Farm A was an intensive pig breeding unit in the west of England, which started with a foundation stock of 6-week-old pigs delivered by hysterectomy from large White and Landrace sows. These animals were received in batches of 22–150 and were housed in new buildings. Two outbreaks of clinical salmonellosis occurred at this farm (Heard, Linton, Penny & Wilson, 1965). The first incident involved two pigs aged 12 weeks. One died after a short illness and the other was destroyed. *S. typhimurium* phage type 1 was isolated from both pigs post-mortem. Rectal swabs of the remaining 62 pigs in the same house showed 44 positive for *S. typhimurium* phage type 1. This organism was also cultured from 22/36 rectal swabs taken at random from pigs in other houses.

Samples of animal feeding stuffs and stool specimens from farm personnel were negative for salmonellas. All animal food was treated for 10 days with nitrofurans (nitrofurazone 25 %, furazolidone 3·6 %) and the 62 pigs were re-examined. Thirty-eight were still positive for the same serotype. Further clinical cases occurred 6 months later when six pigs died over a period of 15 days. *S. typhimurium* phage type 1 was again isolated at post-mortem. No other clinical infections were recorded after treatment with nitrofurans but many pigs remained symptomless salmonella excretors.

Farm A comprised several subunits, sow yard, boar yard, farrowing houses, performance test houses, rearing houses, feed and equipment stores and office. The different pig houses were divided into pens each for one sow (in farrowing house), one to four boars or 8 to 16 pigs. The pens were fitted with troughs and a mains water point. In the sow yard vasectomized boars were allowed to mix with the sows. The sows were taken to the boar yard for service, remaining about 3 hr. Pigs were moved from house to house and pen to pen as they grew and this contributed to the spread of infection.

When infection persisted structural changes were made aimed at controlling spread of disease. Communal dung channels were abolished to prevent faecal contamination between adjacent pens in each pig house. Where possible, underfloor drainage was constructed. Gradient of floors was adjusted to ensure rapid removal of excreta.

Finished and culled pigs from farm A were sent to the bacon factory about a mile away by lorry which was thoroughly disinfected before and after delivery. Pigs were killed without delay to avoid infection of lairages.

The feed used on Farm A at the beginning of the investigation was 'jumbo nuts' and later specially treated pellets.

Farm B was a semi-intensive farm mainly used for fattening a stock of about 300 middle and large white pigs of varying ages and unknown origin. Animals were housed in brick buildings divided into pens for ten or more pigs. The standard of hygiene was reasonably good. Fish meal and barley meal with mineral mixture was fed to the animals. Mains water was available for drinking.

Farms C and D were semi-intensive premises dating from 1965 and the foundation stock of sows came from Farm A. Other stock was obtained from unrelated sources. Details of these farms were similar to Farm B.

Farm E was a minimum disease pig unit. Reared pigs (10–50 including sows and boars) were produced. Specially prepared pellets were fed to these animals.

MATERIALS AND METHODS

Farms

Fresh faecal samples (mostly composite) were collected from floors of the different houses. These were taken with wooden spatulas (one per sample) into waxed containers (Mono). Many were kept at room temperature overnight and examined next day. About 3 g. of well-mixed faeces was suspended in 15 ml. of selenite F broth (Leifson, 1936) and 15 ml. of tetrathionate broth (Rolfe, 1946). Enrichment media were incubated at 37° C. for 24 hr. and subcultured on deoxycholate sucrose citrate and bismuth sulphite agars. Second subcultures were made from negative samples after 72 hr. incubation. One or more suspicious colonies were picked into tubes of Gillies I and II media (Gillies, 1956) and lysine broth and onto MacConkey agar for purity and lactose fermentation. Early in the investigation negative samples were re-examined but the practice was discontinued, as it was found unrewarding. Slide agglutination was performed from Gillies slopes and cultures were finally submitted to the Salmonella Reference Laboratory for identification.

Bacon factory

Mixed caecal and rectal samples were collected from individual Farm A pigs. These were examined by the laboratory attached to the factory. Cultures thought to be salmonellas were sent to the Food Hygiene Laboratory for checking.

At the same time at the bacon factory a parallel survey was conducted on pigs from other farms in various parts of England. Mixed caecal and rectal samples were collected from each of 15–20 pig carcasses at weekly intervals. The pigs were 4 months to 4 years old.

RESULTS

Farm A

This was studied for 2 years. During this period 413/773 (53 %) of faecal samples were positive for salmonellas. The serotypes isolated were *S. bredeney* (148 cultures), *S. durban* (43), *S. heidelberg* (92), *S. manchester* (1), *S. typhimurium* phage type 1 (24), phage type 3*a* (83), phage type 23 (2) and untypable (17), and three unidentified salmonellas.

Four preliminary samples taken at random from floors of the sow yard, farrowing house I, performance test house I and gully trap, receiving effluent from the various pig houses, contained salmonellas. The serotypes were *S. bredeney*, *S. durban*, *S. heidelberg* and *S. typhimurium* phage type 3*a*. A fortnight later 16 pen samples and four drain swabs from gully traps were taken. Six pen samples and one drain swab were positive. The serotypes isolated were *S. bredeney*, *S. heidelberg* and *S. typhimurium*. Further detailed surveys of the different pig houses were carried out.

Many boars excreted more than one serotype, e.g. *S. bredeney*, *S. typhimurium* and *S. heidelberg*. On repeated examination certain boars were found to excrete salmonellas regularly. Others were intermittent excretors.

Table 1 shows the incidence and serotypes of salmonellas grown from faeces from pig houses at Farm A.

Table 1. *Incidence and serotypes of salmonellas grown from faeces from pig houses at Farm A*

Source	No. of samples		Serotypes						
	Examined	Positive	B	D	H	M	T 1	T 3*a*	T 23
Sow house	342	223 (65)	+	+	+	+	+	+	−
Boar house	235	137 (58)	+	−	+	−	+	+	+
Farrowing houses I and II	73	25 (34)	+	−	+	−	+	+	−
Performance test houses I and II	69	8 (12)	+	−	+	−	−	+	−
Rearing houses early and final	31	13 (41)	+	+	+	−	−	+	−

(Figures in parentheses are percentages)

R = *S. bredeney*; D = *S. durban*; H = *S. heidelberg*; M = *S. manchester*; T = *S. typhimurium* (phage types indicated underneath).

Young boars, in groups of eight per pen, were kept in the performance test houses I and II for periods of 14–19 weeks according to their rate of food conversion and weight gain. Pen samples were taken within 2–3 days of their arrival and continued at weekly intervals.

Young boars transferred from the performance test houses, which had a lower incidence of salmonella, into the boar yard were negative during the first few days. After using the central passage and after contact with excretors amongst older boars retained in the herd, the young boars began to excrete salmonellas. Therefore, there was continuity of infection. Batches of in-pig sows were examined within 48 hr. of arrival in the farrowing house.

Piglets were weaned at about 6 weeks and were moved to the early rearing house for about 10 weeks. Boars of good potential were moved to the performance test house, while gilts and rejected boars went to the final rearing house.

A salmonella eradication scheme was instituted 2 years after the start of the investigation. All pigs housed in infected pens were slaughtered. Pens were thoroughly disinfected and restocked with animals from salmonella-free pens. Subsequent examination of 88 faecal samples from all houses collected on two occasions gave only one positive – *S. california*, a serotype not previously found. Six months later all 121 faecal specimens examined on three occasions were negative for salmonellas.

Farm B

Preliminary examination of pen samples from five pens showed 1/5 specimens positive; *S. schwarzengrund* was isolated. Later 14 samples were examined from 14 pens, including those first investigated. *S. schwarzengrund* was again found in the same pen.

A boar known to be an intermittent excretor of *S. bredeney* was introduced into the herd from Farm A. The animal was first isolated for 6 weeks and faeces collected daily for the first 6 days; the specimen taken on the fourth day contained *S. bredeney*. After isolation the boar was allowed to mix with the rest of the herd including pigs from the pen positive for *S. schwarzengrund*. Four weeks later samples were examined from the boar and from 11 other pens. The boar was negative but the incidence of salmonella excretion had increased in the other pigs. Six pen samples contained *S. schwarzengrund* but *S. bredeney* was not found.

Farm C and Farm D

Foundation stocks for both farms came from Farm A. Sampling at Farm C began about 10 months after arrival of pigs. Fifty-eight faeces were tested on three occasions (8, 10 and 40 samples) at four-week intervals. Salmonellas were not isolated at Farm C. Eleven faeces samples collected from pens 5 months after arrival of pigs at Farm D contained *S. bredeney* and an unidentified salmonella in four of the samples. *S. bredeney* and an unidentified serotype were found in the sow yard at Farm A.

Farm E

Batches of 6-week-old pigs produced by hysterectomy on this unit formed the foundation stock for Farm A. Six faecal samples from sows and litters on four occasions were examined with negative results.

Bacon factory

Mixed rectal and caecal faeces from 1053 pigs from Farm A were cultured. Three hundred and thirty-four (32 %) contained salmonellas; serotypes isolated were *S. bredeney*, *S. durban*, *S. heidelberg*, *S. orion* and *S. typhimurium* phage types 1 and 3*a*. *S. orion* had been found on Farm A at an earlier date.

Pigs from other sources were also examined at the bacon factory. Results of faecal cultures of animals from various farms in different counties were compared with those from Farm A. Thirty-four out of 586 (6 %) specimens contained salmonellas (Table 2). Fourteen serotypes were found: *S. anatum*, *S. alachua*, *S. bredeney*, *S. canoga*, *S. give*, *S. heidelberg*, *S. litchfield*, *S. livingstone*, *S. manchester*, *S. newport*, *S. panama*, *S. senftenberg*, *S. stanley* and *S. typhimurium* phage types, 9, 12*a*, U 84 and U 184.

Information on housing and feeding methods at the farms was not obtained. It was known, however, that pelleting and heat treatment of feeds as used on Farm A were not applied elsewhere in the locality. It is of interest that many serotypes isolated were different to those found on Farm A. Feeding stuffs were suspected as the source of these strains.

DISCUSSION

Symptomless excretor animals are potentially important in the spread of infection. In the present investigation it was observed that the distribution of symptomless excretors from pig-breeding farms resulted in the transmission of infection to rearing establishments.

Faecal samples from pens were more reliable specimens for salmonella isolation than rectal swabs from single pigs (Bevan Jones *et al.* 1964). Rectal swabs provide only a scanty amount of faeces for culture owing to contraction of the sphincter muscles, possibly more pronounced in nervous animals.

During the 2-year investigation, four different serotypes were persistently found on one farm: *S. bredeney*, *S. durban*, *S. heidelberg* and *S. typhimurium* phage types 1, 3*a*, 23 and untypable. *S. manchester* was isolated once from the sow house and *S. typhimurium* phage type 23 twice from the boar house. Other serotypes were fairly regularly cultured. With the exception of *S. durban* and *S. manchester*, all serotypes isolated on Farm A have been found in animal feeding stuffs (Taylor *et al.* 1965).

Although salmonellas were not isolated from feeds on the farm during the investigation, feeds were thought to be a probable source of infection at some earlier time. Measures were taken to prevent re-introduction of salmonellas by feeding pellets prepared at high temperatures. Absence of new serotypes during the 2-year period suggest that this may have been successful. The comparative

study at the bacon factory of faecal samples from pigs reared on different feeds at other farms showed the existence of other sources of salmonellas entering the factory (Table 2). Edel, Guinée, van Schothorst & Kampelmacher (1967) found that the use of pelleted feeds controlled infection. The high cost of pellets may be offset by a saving in food material.

Although the introduction of further infection into Farm A was prevented, the programme of breeding necessitated movement of pigs and encouraged dissemination and perpetuation of infection through the various pig houses. Symptomless excretors, mainly sows and boars, were responsible for the spread of disease, as they were retained for lengthy periods on Farm A. Most positive samples came from sow and boar yards (Table 1). Sows and vasectomized boars mixed freely. Sows were moved around frequently and vasectomized boars were regularly positive for salmonellas.

In the boar yard the central passage used for exercise probably helped to spread infection. Sows retained for service with boars might become infected or pass on infection. Some boars with excellent genetic potential were found to be regular excretors. Young boars could be infected by older boars through stay in contaminated environments.

Samples from farrowing houses, where sows were kept singly in pens, and from early and final rearing houses showed a lower incidence of infection than those in the other houses. Young animals are regarded as being more susceptible to

Table 2. *Incidence and serotypes of salmonellas from caecal and rectal contents of pigs at the bacon factory*

Source of pigs	No. of pigs		*Salmonella* serotypes	Source of pigs	No. of pigs		*Salmonella* serotypes
	Examined	Positive			Examined	Positive	
Berks.	10	0	—	Herts	6	0	—
Bristol	22	1	*stanley*	Kent	53	5	*panama*
Cornwall	45	1	*anatum*				*typhimurium* U 84
Devon	31	0	—	Norfolk	8	1	unident.
Dorset	21	0	—	Somerset	98	12	*bredeney* *livingstone*
Essex	42	3	*give* *typhimurium* 9				*panama* *typhimurium* U 184
Glos.	46	4	*litchfield* *newport* *typhimurium* 12*a*	Suffolk	13	0	—
				Surrey	9	2	*manchester*
				Sussex	26	0	—
Hants	26	4	*alachua* *canoga* *senftenberg* *typhimurium*	Wilts	30	0	—
				Unknown	100	1	*heidelberg*

Unident. = unidentified.

The figures under the *S. typhimurium* strains indicate phage types.

infection (Buxton, 1957) and the absence of clinical disease among young piglets may be explained by the fact that they were kept in separate houses and did not come into contact with older boars and sows except for a short period after birth. Colostrum immunity might also be acquired.

Infection by the movement of excretor pigs from farm to farm was demonstrated by transference of animals from Farm A to Farm D which was newly constructed and housing pigs for the first time.

Foundation stock from Farm D included a boar regularly excreting *S. bredeney*, *S. heidelberg* and *S. typhimurium* and a number of sows excreting unidentified salmonellas. Subsequent tests showed infection with *S. bredeney* and unidentified strains persisted not only in boars and sows but also in offspring. Stress factors must be taken account of during transport as these may encourage excretion of salmonellas (Williams & Newell, 1970).

Spread of infection through excretor pigs was also recorded on Farm B. Initial examination showed that samples from one pen contained *S. schwarzengrund*. Isolation of this serotype from animal feed was reported by Taylor *et al.* (1965). In the investigation described, serotypes found in pigs on the farm and in the bacon factory corresponded to those isolated from feeding stuffs. Another survey in the south west of the United Kingdom has given similar results (PHLS Working Group *et al.* 1972). The situation in Britain was contrasted in this study with that in Denmark where feed ingredients of animal origin are terminally heat treated to eliminate salmonellas. This has reduced the prevalence of types other than *S. typhimurium* in pigs in Denmark, as compared with England. Contaminated feeding stuffs are known to initiate symptomless excretion in pigs (Smith, 1960). Meat may become contaminated by faeces during slaughter and it is then a potential vehicle for infecting man.

Stool samples from farm personnel and rodents were negative for salmonellas. The role of wild animals and birds as primary infection sources is debatable. They are usually accepted as being victims of a contaminated environment.

The more animals excreting salmonellas when they leave the farm the greater the build up of infection during transport and more widespread the environmental contamination in the abattoir. The longer the holding period of animals before slaughter the greater is the danger of spread of disease (Galton *et al.* 1954; McDonagh & Smith, 1958; Anderson, Galbraith & Taylor, 1961; Bevan Jones *et al.* 1964).

Implementing effective control measures against human and animal salmonellosis involves taking adequate steps to prevent transmission of infection through animals, detecting and eliminating symptomless excretors from farms and preventing introduction of fresh infection, for example, through contaminated feeding stuffs. The institution of efficient feed sterilization methods could contribute to this control.

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Vibriocidal antibodies induced by *Yersinia enterocolitica* serotype IX

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SUMMARY

Yersinia enterocolitica serotype IX has been found to stimulate the production of vibriocidal antibody against *Vibrio cholerae*, particularly in Inaba serotype, in experimental rabbits and infected man to a significant degree. This activity could be absorbed by both Inaba and *Yersinia* antigens from anti-yersinia sera, but *Yersinia* antigen could not absorb vibriocidal activity from the anti-cholera sera, indicating a unilateral relation.

Vibrio agglutinating antibody, particularly against Ogawa, in anti-yersinia rabbit or human sera was found to be less liable to non-specific stimulation.

V. cholerae, while removing vibriocidal antibody, could not absorb the *Yersinia* agglutinin from anti-yersinia sera, suggesting that there is a different determinant for these two types of antibody activities.

The non-specific production of vibriocidal antibody by *Y. enterocolitica* type IX indicates the need for caution in the interpretation of the results of vibriocidal tests where such organisms are prevalent.

INTRODUCTION

Yersinia enterocolitica is not a very well known organism and has only recently been differentiated from *Pasteurella pseudotuberculosis* on the basis of biochemical and serological tests. It was referred to as *Pasteurella* X by Knapp & Thal (1963) and Carlsson, Ryd & Sternby (1964), but Frederiksen (1964) proposed the designation of *Y. enterocolitica*. It is known to occur in animals such as chinchillas, hares, pigs and dogs, and has also been suspected of causing terminal ileitis, mesenteric lymphadenitis (clinically simulating acute appendicitis), diarrhoea and erythema nodosum in man (Winblad, Niléhn & Sternby, 1966; Niléhn, 1967; Ahvonen & Sievers, 1969). In 1967, Winblad described eight serotypes of *Y. enterocolitica* based on O antigens, but his collection of 105 strains did not include the serotype which cross-reacts strongly with *Brucella* species and which was described by Ahvonen & Sievers (1969) as *Y. enterocolitica* serotype IX.

Tests for vibriocidal and agglutinating antibodies have been increasingly employed in recent years in studies on the immunology and epidemiology of

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cholera. The presence of vibriocidal antibody in the sera of American citizens who have never been vaccinated against cholera nor exposed to cholera infection (Finkelstein, Powell, Woodrow & Krevans, 1965; Verwey *et al.* 1969) has raised the question of the specificity of the vibriocidal reaction. Cross-reactivity between *Vibrio cholerae* and *Brucella* species is widely known (Eisele, McCullough, Beal & Burrows, 1946; Gallut, 1950; Mathur, 1960) and has recently been again emphasized by Feeley (1969) and Gangarosa, DeWitt, Feeley & Adams (1970), who found other instances of the presence of vibriocidal antibody in human sera in the absence of exposure to cholera infection. Corbel & Cullen (1970) have also recently demonstrated cross-reacting antibodies induced by *Br. abortus* and *Y. enterocolitica* serotype IX in cattle by various techniques.

Infection by *Y. enterocolitica* appears to be fairly common in countries where attempts are made to look for the organism. Recently Makulu, Gatti, Mollaret & Vandepitte (1969) reported 11 isolations in 8 months in the Democratic Republic of the Congo.

This investigation was undertaken to elucidate the possible antigenic relationship between *V. cholerae* and *Y. enterocolitica* serotype IX which may have an influence on sero-epidemiological studies.

METHODS AND MATERIALS

Strains and sera

Strains of *V. cholerae*, Ogawa NIH 41 and Inaba V 86, from the lyophilized stock culture in this laboratory were used to prepare antigens for the immunization of animals as well as for antibody titrations.

Strain M.Y. 79 of *Y. enterocolitica* serotype IX and nine human sera from bacteriologically confirmed cases of infection by this organism were kindly provided by Dr P. Ahvonen, Helsinki, Finland. In this communication the term '*Yersinia*' refers to this particular serotype.

Immune sera against *V. cholerae* Ogawa and Inaba were prepared in rabbits (four for each serotype) by injecting live saline suspensions from a 7 hr.-old growth (at 37° C.) on heart infusion agar containing about 2×10^9 organisms per ml. Each of the rabbits received three intravenous injections, 5 days apart, of 0·25, 0·5, and 0·5 ml.; they were bled seven days after the last injection and the sera were pooled as Ogawa and Inaba.

Four rabbits were used to prepare antisera against *Y. enterocolitica* serotype IX. Two of them received two intravenous injections, seven days apart, of 0·25 and 0·5 ml. of live saline suspension from a 48 hr.-old growth (at about 22° C.) on heart infusion agar containing about 2×10^9 organisms per ml. Identical suspensions were heated at 120° C. for two hours and similarly injected into two other rabbits. Blood was collected 7 days after the second injection. Sera against the live and heated antigens were pooled separately and all the sera were stored at −20° C.

Agglutination test

Heated antigen was preferred to live antigen as this permitted repetition of the tests using the same antigen. Saline suspensions from a 4 hr.-old growth of *V. cholerae* on heart infusion agar at 37° C. were heated at 100° C. for 1 hr. for the preparation of Ogawa and Inaba antigens. Forty-eight-hour-old growth of *Y. enterocolitica* (as it grows slowly) on heart infusion agar at about 22° C. was suspended in saline and heated at 100° C. for 2 hr. for the preparation of *Yersinia* antigen. These suspensions were washed once in saline and then adjusted to Klett–Summerson colorimeter reading 30 (OD 0·061) using a green filter.

Equal volumes of the antigens were added to 0·5 ml. volumes of the serial 2-fold dilutions of the sera in normal saline in round-bottomed 10 × 90 mm. tubes and then placed in a 52° C. water bath overnight.

Tests were read with the naked eye; the highest dilutions showing fine but definite clumps and clearing of the suspending medium constituted the end-point.

Antisera were also tested in the same way with saline suspensions of live *V. cholerae* Inaba and Ogawa and *Yersinia* grown on heart infusion agar after standardizing the suspensions to the same optical density.

Vibriocidal test

The test was done as previously described by Verwey *et al.* (1969). Serial 2-fold dilutions of the sera in Kolmer's saline in 0·2 ml. volumes were mixed with equal volumes of the complement – cell mixture (complement 1/10 (Hyland Lab.); *V. cholerae* Inaba or Ogawa, 2×10^8 per ml.) and incubated in a 37° C. water bath for 1 hr. with occasional shaking. Brain heart infusion broth, 1·6 ml., was then added to all tubes which were incubated again for 2–2½ hr. The tubes were then examined with the naked eye for the amount of growth of *V. cholerae* as compared with that in the control tube; the degree of turbidity was graded from 0 to 4+, and a 2+ growth was considered as the 50% end-point. Appropriate controls and reference sera were included in each test.

A Brewer machine was used for distribution of the vehicle and for adding broth, and an automatic serum diluter (American Instrument Co.) was used for serum dilution.

The results of the agglutinating and vibriocidal tests are expressed in reciprocals of the titres.

Absorption of the sera

The antigens were prepared in the same way as for the agglutination test, but the suspension contained 1×10^{11} organisms per ml. In the first stage of absorption, 0·5 ml. of the antigen was diluted with 5 ml. of saline and centrifuged at 12,100 ***g*** for 30 min. The bacterial deposit was carefully mixed with 5 ml. of a 1/5 dilution of the serum to be absorbed. The mixture was incubated at 37° C. in a water bath for 2 hr. with occasional shaking, and then centrifuged at 26,384 ***g*** for 30 min. For the second absorption, this supernatant was mixed with the bacterial mass from 2·5 ml. of antigen suspension diluted with 5 ml. of saline and centrifuged at

12,100 *g* for 30 min. The mixture was incubated again in a 37° C. water bath for 2 hr. with occasional shaking and then kept at 4° C. overnight before being centrifuged at 26,384 *g* for 30 min. The supernatant was collected and stored at − 20° C.

RESULTS

Y. enterocolitica serotype IX strain M.Y.79 did not react well in the complement-dependent bactericidal system with the homologous or anti-*V. cholerae* sera. In the agglutination test the heated O antigen of this organism was found to react better than live suspension; antisera prepared by injecting live or heated *Yersinia* did not produce any visible clumping with live homologous antigen, as shown in Table 1. This table also shows that anti-cholera sera agglutinated heated *Yersinia* antigen to a titre of only 40–80 and did not react at all with *Yersinia* live antigen. Anti-yersinia sera, on the other hand, exhibited very high vibriocidal activity against Inaba serotype; two out of the four rabbits, one receiving live and another heated antigen, also developed Ogawa vibriocidal titre to a moderate degree. One of the four anti-yersinia sera revealed some agglutinin titre against heated Inaba and Ogawa antigen; all of them had some agglutinating activity against live Inaba (OH) antigen.

The results after two-step absorption of the anti-cholera and anti-yersinia sera with heated *V. cholerae* (Ogawa and Inaba) and *Yersinia* antigen are shown in Table 2. The titres of the agglutination test appear to be different in this table from those in Table 1, because the initial dilution of the sera in this case was 1/15. Table 2 shows that the group-specific vibriocidal and agglutinating activity of anti-cholera sera could be removed by absorbing the anti-Inaba serum with Ogawa antigen and anti-Ogawa serum with Inaba antigen in appropriate quantities

Table 1. *Vibriocidal and agglutinating antibody titre of immune rabbit sera*

	Vibriocidin titre		Agglutinin titre					
			Inaba		Ogawa		*Y. ent.* (IX)	
	Inaba	Ogawa	Live	Heated	Live	Heated	Live	Heated
V. cholerae Inaba–Live (K)	163,840	163,840	2,560	2,560	2,560	1,280	–	80
V. cholerae Ogawa–Live (J)	327,680	327,680	1,280	1,280	5,120	1,280	–	40
Y. enterocolitica Serotype IX Live (75)	10,240	1,280	40	–	–	–	–	10,240
Live (73)	2,560	20	40	–	–	–	–	2,560
Y. enterocolitica Serotype IX Heated (77)	40,960	20	160	–	–	–	–	2,560
Heated (80)	5,120	1,280	40	20	–	80	–	2,560

Pre-immunization vibriocidal titres against Ogawa and Inaba were less than five in all animals except Nos. 80 and 75 in which the titres against Inaba were ten and five respectively.
'–' = less than 10.

but not by the *Yersinia* antigen. Inaba vibriocidal activity in the anti-yersinia sera could be removed by Inaba antigen but not by Ogawa, and neither of the two vibrio antigens could reduce homologous *Yersinia* agglutinin titre. Ogawa vibrio-

Table 2. *Antibody titre of immune rabbit sera before and after absorption with heated homologous and heterologous antigens*

(Agglutinating titre with heated antigens only)

		Vibriocidin titre		Agglutinin titre		
Sera	Absorbed with	Inaba	Ogawa	Inaba	Ogawa	*Y. ent.* (IX)
V. cholerae	–	163,840	163,840	3,840	3,840	60
Inaba (K)	Inaba	20	–	–	–	–
	Ogawa	81,920	1,280	1,920	–	–
	Y. ent. (IX)	163,840	163,840	1,920	1,920	–
V. cholerae	–	327,680	327,680	960	1,920	60
Ogawa (J)	Inaba	40	81,920	–	1,920	–
	Ogawa	–	40	–	–	–
	Y. ent. (IX)	163,840	163,840	960	960	–
Y. ent. (IX)	–	40,960	20	–	–	–
Heated (77)	Inaba	–	–	–	–	3,840
	Ogawa	20,480	–	–	–	3,840
	Y. ent. (IX)	–	–	–	–	3,840
Y. ent. (IX)	–	10,240	1,280	–	–	7,680
Live (75)	Inaba	–	40	–	–	3,840
	Ogawa	10,240	–	–	–	7,680
	Y. ent. (IX)	–	–	–	–	–

Vibriocidin titre: '–' = less than 10.
Agglutinin titre: '–' = less than 30.

Table 3. *Antibody titre of immune rabbit sera before and after absorption of anti-*V. cholerae *sera with live* Y. enterocolitica *antigen*

(Agglutinating titre with heated antigens only)

		Vibriocidin titre		Agglutinin titre		
Sera*	Absorbed with	Inaba	Ogawa	Inaba	Ogawa	*Y. ent.* (IX)
Inaba (K)	–	163,840	163,840	2,560	1,280	80
Inaba (K)	*Y. ent.*	163,840	81,920	2,560	1,280	80
Ogawa (J)	–	327,680	327,680	1,280	1,280	40
Ogawa (J)	*Y. ent.*	327,680	327,680	1,280	1,280	40
Monovalent Inaba	–	2,560	80	40	–	–
Monovalent Inaba	*Y. ent.*	2,560	80	40	–	–
Monovalent Ogawa	–	80	5,120	–	160	–
Monovalent Ogawa	*Y. ent.*	80	5,120	–	160	–

* Inaba (K) and Ogawa (J) sera were unabsorbed anti-cholera sera containing both group and type antibodies.

Monovalent Inaba and Ogawa sera were absorbed by heterologous antigens and, therefore, type-specific.

'–' = less than 10.

cidal titre seen in one anti-yersinia serum was absorbed by Ogawa antigen. Absorption with *Yersinia* antigen of the homologous sera removed both the heterologous vibriocidal and the homologous agglutinating activity almost completely.

Table 3 shows that the live *Yersinia* antigen, like the heated antigen, could not absorb the vibriocidal and agglutinating antibodies of polyvalent anti-cholera sera (with both group and type-specific antibodies) nor those of monvalent Inaba and Ogawa sera (with type-specific antibody only) kindly supplied by Dr H. Smith of the U.S. Vibrio Reference Center.

Table 4. *Vibriocidal and agglutinating activities of sera from patients with infection by* Y. enterocolitica *serotype IX*

	Vibriocidin titre		Agglutinin titre (heated antigen)		
	Inaba	Ogawa	Inaba	Ogawa	*Y. ent.*
1 *(2215)	–	–	–	–	–
2 (3208)	640	320	160	–	320
3 (1287)	10,240	160	160	–	160
4 (954)	20	80	–	–	640
5 (112)	320	40	40	40	320
6 (3649)	1,280	640	40	–	320
7 (4381)	160	40	–	–	160
8 (2207)	20	–	–	–	40
9 (1246)	5,120	40	–	–	80
10 (1159)	5,120	20	–	–	1,280

* Serum from a patient infected with *Y. enterocolitica* of a different serotype.
'–' = less than 20.

Table 5. *Vibriocidal and agglutinating antibody titre of sera from patients with infection by* Y. enterocolitica *serotype IX*

(After homologous and heterologous absorption with heated antigen.)

Sera	Absorbed with	Vibriocidin titre		Agglutinin titre		
		Inaba	Ogawa	Inaba	Ogawa	*Y. ent.*
2	–	640	320	160	–	320
	Yersinia	40	–	–	ND	–
	Inaba	–	–	–	ND	320
3	–	10,240	160	160	–	160
	Yersinia	–	–	–	–	–
	Inaba	–	–	–	ND	160
6	–	1,280	640	40	–	320
	Yersinia	–	–	ND	ND	ND
9	–	5,120	40	–	–	80
	Yersinia	–	–	–	ND	–
	Inaba	–	–	–	ND	80
10	–	5,120	20	–	–	1,280
	Yersinia	–	–	–	ND	–

'ND' = not done.
'–' = less than 20.

The results of the vibriocidal and agglutination tests on ten human sera are shown in Table 4; nine of them were from bacteriologically confirmed cases of *Y. enterocolitica* serotype IX. Serum No. 2215 from a patient with infection by *Y. enterocolitica* of a different serotype did not show any demonstrable antibody against *Y. enterocolitica* serotype IX or *V. cholerae* in tests employed. Of the nine other sera, all of which had *Yersinia* agglutinin titres varying from 40 to 1280, seven had high vibriocidal activity against Inaba; the Ogawa vibriocidal titre was less pronounced. Agglutinin titre with *V. cholerae* was not remarkable in most of the sera, particularly against Ogawa.

The absorption of five of these sera with heated antigens of Inaba and *Yersinia* again revealed that *V. cholerae* Inaba antigen could remove the vibriocidal activity but not the *Yersinia* agglutinating activity whereas *Yersinia* antigen could absorb both the activities (Table 5).

DISCUSSION

The results of this study show that *Y. enterocolitica* serotype IX induces the production of vibriocidal antibody, particularly against serotype Inaba, both in the rabbit and in man. This was not unexpected in view of the previous reports on cross agglutination between *Y. enterocolitica* serotype IX and *Brucella* species (Ahvonen & Sievers, 1969; Ahvonen, Jansson & Aho, 1969) and also between *Brucella* species and *V. cholerae* (Feeley, 1969; Gangarosa *et al.* 1970). However, in the present study, the antigenic relationship between *Yersinia* and *V. cholerae* appeared to be rather complex.

It was not possible to perform classical absorption studies of the antisera for the determination of antigenic relationship between the relevant organisms for the following reasons:

(*a*) Two high titre anti-cholera sera agglutinated heated *Yersinia* antigen to only a low titre of 40–80 (Table 3) which was absorbed by heated Inaba, Ogawa and also *Yersinia* (Table 2) but not by live *Yersinia* antigen (Table 3). All antisera, including those produced by injecting live *Yersinia* antigen, failed to agglutinate live suspension of *Yersinia* probably because of the presence of a surface antigen (Table 1).

(*b*) None of the four anti-yersinia sera reacted in the agglutination test with heated *V. cholerae* antigen: agglutinin titre against live Inaba antigen was low, 40 in three sera and 160 in one (Table 1).

(*c*) As mentioned earlier, *Yersinia* and cholera antisera could not be tested for bactericidal activity against *Y. enterocolitica* to examine whether *V. cholerae* produced bactericidal antibody against *Yersinia* in the same way that *Yersinia* induced vibriocidal antibody.

In spite of these limitations, it could be shown by the absorption studies that while the Inaba vibriocidal antibody in anti-yersinia rabbit sera could be removed by *V. cholerae* Inaba (but not Ogawa) as well as by *Yersinia*, this same activity could not be absorbed from the anti-cholera sera by *Yersinia* cells. It is also interesting to note that Inaba antigen removed the vibriocidal activity from anti-yersinia sera without affecting the *Yersinia* agglutinating activity of the same

sera, indicating different antigenic determinants in *Yersinia* for these two different types of antibodies. When anti-yersinia sera were absorbed with *Yersinia* antigen, both the vibriocidal and *Yersinia* agglutinating antibodies were removed (Table 2). These observations were also confirmed by absorption of five human sera (Table 5), although complete cross absorption studies of these sera could not be done because of the limited amount of sera available.

The results of the present study also indicate that the antigen responsible for vibriocidal antibody formation in *Yersinia enterocolitica* serotype IX is heat-stable as in the case of *V. cholerae*, though the heated, like the live *Yersinia* antigen was ineffective in absorbing the vibriocidal activity in the anti-cholera sera. This type of non-reciprocal relationship was also described between the O antigens of *Sh. dysenteriae* 4 and *E. coli* O group 88 and between the O antigens of *Sh. dysenteriae* 1 and *E. coli* O group 120 by Ewing (1953) on the basis of agglutination reaction; no satisfactory explanation was offered for this phenomenon.

Heterophile antibodies might have explained these peculiar serological reactions, and all antisera prepared in rabbits were tested and found negative for agglutinin against sheep red cells by the technique of Davidsohn & Walker (1935).

In view of the fact that the cross-reacting antibody produced by *Yersinia* was mostly vibriocidal in nature against Inaba and that Inaba agglutinating antibody was found in 4 out of 9 and Ogawa agglutinating antibody in 1 out of 9 sera from human *Yersinia* infection, it appears that the somatic agglutinin against *V. cholerae* is less liable to non-specific stimulation as far as *Y. enterocolitica* serotype IX is concerned.

The present observations indicate that the results of sero-epidemiological surveys and immunological studies for cholera based on vibriocidal antibody titration should be interpreted with caution, particularly in areas where organisms serologically cross-reacting with *V. cholerae*, including *Y. enterocolitica* type IX, are prevalent.

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Comparative complement-fixation studies with subtype strains of foot-and-mouth disease virus

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SUMMARY

A comparison was made between a macrotechnique in tubes and a microtechnique in plastic plates for complement-fixation tests, using strains of three subtypes of the Asia 1 type of foot-and-mouth disease (FMD) virus. The results obtained with these techniques were found to be comparable and delineated the antigenic relationships of the three strains employed. The microtechnique was considered to be both economical with reagents and capable of similar accuracy and reproducibility to that of the standard method in tubes. It was concluded that the antigenic analysis of subtype strains of FMD virus can be conveniently carried out by the use of the microtechnique as described.

INTRODUCTION

Complement-fixation reactions using foot-and-mouth disease (FMD) virus systems have been studied extensively (Traub & Möhlmann, 1946; Brooksby, Galloway & Henderson, 1948; Brooksby, 1952; Graves, 1960). Such studies have generally been made by means of macrotechniques which require relatively large volumes of each reagent. An economical use of reagents with certain other virus systems has been achieved when the reagent volumes have been reduced to comparatively small amounts; for example, with influenza (Fulton & Dumbell, 1949; Fulton, 1951), bluetongue (Kipps, 1956) and Teschen viruses (Darbyshire & Dawson, 1963).

The application of similar microtechniques to FMD virus systems has not been recorded previously. The following paper describes the application of a microtechnique of complement fixation to the investigation of antigenic relationships of strains of three subtypes of the Asia 1 type of FMD virus. At the same time, a detailed comparison was made of such a method with an existing technique done in tubes and previously used to identify both type and subtype differences of FMD virus (Brooksby, 1952; Davie, 1964).

MATERIALS AND METHODS

Antigens

Three strains of Asia 1 type FMD virus were investigated. The strains were obtained from the collection of the World Reference Laboratory and were, respectively, Pak 1/54 (a Pakistan strain which was the first recognized Asia 1 type), Isr 3/63 (an Israeli field strain from Yokneam), and Isr 1/57 (the first Asia 1 type recognized in Israel in 1957). The antigens were each produced in one of several different systems as described, namely:

Pig kidney cells. Monolayer cultures of the IB-RS-2 (clone 60) cell line of porcine kidney (de Castro, 1964) were produced in bottles, using a growth medium consisting of Hanks's saline, 3% lactalbumin hydrolysate, antibiotics (neomycin, 65 i.u./ml., mycostatin, 60 i.u./ml. and penicillin, 600 i.u./ml.) and 10% ox serum. The cultures were washed, inoculated with virus and allowed to adsorb for 30 min. at 37° C. They were then overlaid with a maintenance medium consisting of Earle's saline with 5% lactalbumin hydrolysate and antibiotics as above, left for up to 48 hr. and harvested when approximately 90% of the cell sheet showed a cytopathic effect. Cell debris was removed by low-speed centrifugation and the supernatant used as antigen.

Baby hamster kidney cells. Monolayer cultures of baby hamster kidney cells were prepared with the BHK 21 (clone 13) cell line. The cultures were prepared and used as described above.

Guinea-pig materials. Guinea-pigs, weighing 500–700 g., were inoculated intradermally into the metatarsal pads with virus. Vesicular lesions developed within 24 hr. The vesicle epithelium and fluid were subinoculated into further guinea-pigs at the same site and after several such passages the epithelium and vesicle fluids were each subsequently harvested to provide pad epithelium and vesicle fluid antigens, respectively. The pads were ground in 0·04 M phosphate buffer, pH 7·6, to give a 1/10 suspension which was clarified by low-speed centrifugation. The vesicle fluid was also clarified and was suitably diluted for use.

Antisera

The antisera were available from the World Reference Laboratory and had been prepared in guinea-pigs as described previously (Davie, 1964). After approximately 4 weeks, immune guinea-pigs were re-inoculated intradermally with a suspension of fresh infected pads (1/5) or tissue culture virus with saponin as adjuvant. Serum was collected 10 days later, pooled, filtered through a Seitz EK filter and stored at −20° C. until required.

Complement

The complement was normal guinea-pig serum. A number of adult guinea-pigs were starved for 24 hr. and the pooled serum collected and treated with Richardson's preservative (Richardson, 1941) before storage at 4° C. in suitable volumes. During all tests the range of dilutions of complement was kept in an ice bath.

Haemolytic indicator system

The indicator system used in the tests performed in plastic plates consisted of a 0·7 % suspension of washed sheep red cells previously sensitized with an equal volume of a 1/800 dilution of haemolytic equine serum (Burroughs Wellcome & Co.). The haemolytic indicator system used in the tests carried in tubes was a 1·5 % sensitized suspension of sheep red cells. In each case the dilution of haemolysin chosen ensured excess and was the equivalent of at least four haemolytic doses.

Complement-fixation tests

Two techniques of performing complement-fixation tests were investigated and these were done simultaneously, using the same reagents for each method. All the antisera were first inactivated by heating at 56° C. for 30 min.

The first technique essentially followed that developed originally by Brooksby (1952) using test tubes. The practical details of the test have been described (Davie, 1964) and were two-dimensional titrations of complement against antisera in the presence of a fixed dilution of antigen. Suitable controls were incorporated in each test and all reagents were maintained at 4° C. when the tests were being set up. Antiserum, complement and antigen mixtures were incubated in a water bath at 37° C. for 30 min. The indicator system was added (1 ml.) to each tube and the tubes re-incubated at 37° C. for a further 30 min. All the tubes were briefly centrifuged and the degree of haemolysis determined as the optical density of the released haemoglobin in a colorimeter.

The second method was performed in disposable plastic micro plates (Cooke Engineering Co., U.S.A.). For the tests in the microplates 5 volumes of 25 μl. each were used. The procedure of the test was essentially that of Fulton & Dumbell (1949) and Fulton (1951). Two-dimensional titrations, varying both complement and antiserum, were done in the presence of a fixed dilution of antigen and full controls were incorporated in each test. The antiserum, complement and antigen mixtures were incubated at 37° C. for 30 min., during which the plates were covered to reduce evaporation. The haemolytic indicator system was then added to each well as a 50 μl. drop and the plates sealed with transparent tape and re-incubated at 37° C. for 30 min.; the plates were gently agitated from time to time to resuspend any unlysed red cells in the mixtures. The plates were then centrifuged at 500 g for 5 min. to deposit unlysed red cells and examined.

In the microtechnique, the degree of lysis in the indicator system was estimated from the pattern of unlysed cells deposited in each well. The complement dilution interval was 0·18 $\log_{10}$ and the transition of complete lysis to no lysis in any particular row was sufficiently rapid to enable the point of 50 % lysis to be conveniently determined by inspection or, where necessary, by interpolation.

RESULTS

The method of calculating the results obtained with the test in tubes and adopted here has been given in principle by Davie (1964). In the microtechnique the amount of complement fixed by each serum dilution in the presence of antigen

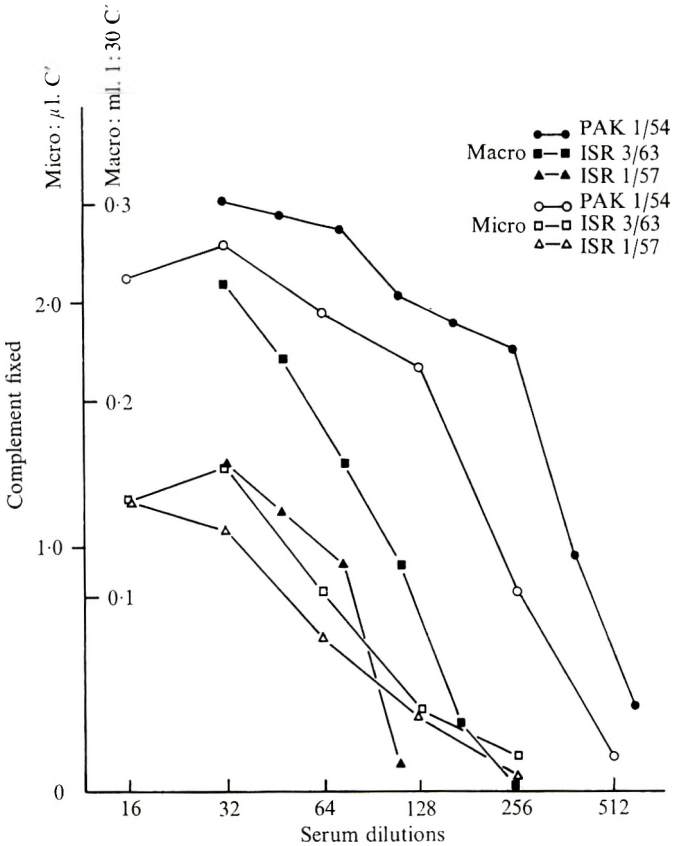

Fig. 1. Complement-fixing activity of three strain-specific antisera with a guinea-pig vesicular fluid antigen (Pak 1/54) using two methods.

was calculated after Fulton (1951). For each method, the amounts of complement fixed by antigen and a particular antiserum were drawn graphically; an example is given in Fig. 1.

It will be seen that the general shapes of the curves for both methods are comparable. In those parts of the curves which approached linearity, especially in the range of the higher serum dilutions, the amounts of complement fixed were used to calculate a mean value of the reaction slope. For this, the method proposed by Bradish, Brooksby & Tsubahara (1960) was adopted, using the formula

$$B = \frac{V_c - V_0}{v_i},$$

where V_c is the total volume of complement required for 50 % lysis, V_0 is the volume of complement required by the anticomplementary reagents, and v_i is the volume of serum used in the test over the dilution series employed. In this manner, the value of the amount of complement which could be fixed by each antigen with undiluted antiserum was calculated.

The values obtained when each antigen was tested against either the homologous or the heterologous antisera in turn are illustrated in Fig. 2, and represent the

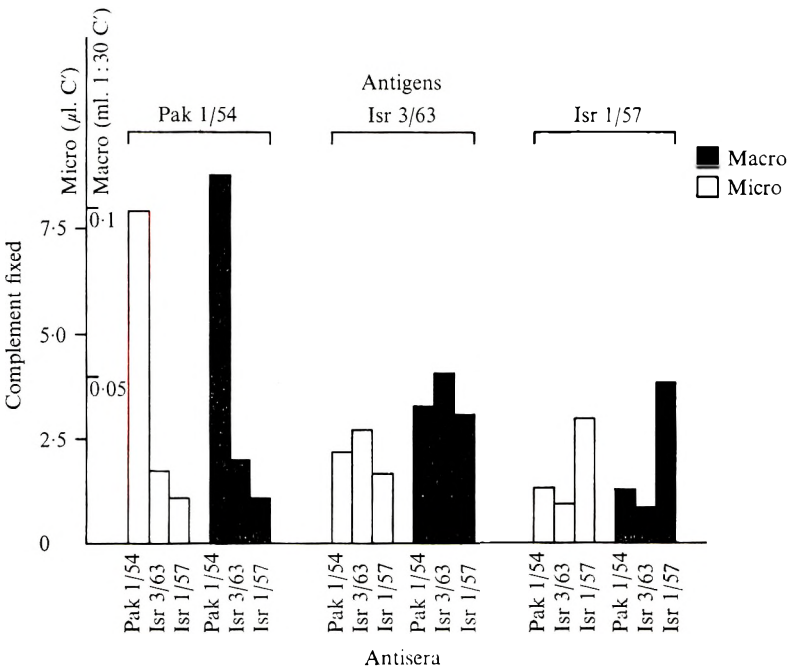

Fig. 2. Complement-fixing activity of three guinea-pig vesicular fluid antigens with homologous and heterologous antisera using two methods.

differences observed in reactivity between pairs of antigens and antisera when using either technique. The amounts of complement fixed by such pairs of reagents were used to assess the antigenic relationships between the virus strains by expressing each homologous and heterologous reaction as a ratio. Thus, for example, using antigens A and B with homologous antisera a and b, a ratio, r_A for antigen A would be represented by

$$r_A = \frac{Ab}{Aa},$$

where Ab represents the complement-fixing activity of the heterologous reaction and Aa the complement-fixing activity of the homologous reaction. Similarly, a ratio for antigen B would be derived from

$$r_B = \frac{Ba}{Bb}.$$

The antigenic relationship (R) of the two antigens may be expressed from the product of such ratios as a percentage, by use of the following formula (Archetti & Horsfall, 1950; Chu, Andrewes & Gledhill, 1950; Jordan & Gaylin, 1953; Wenner, Kamitsuka & Lenahan, 1956; Ubertini *et al.* 1964)

$$R = 100\sqrt{(r_A r_B)}.$$

Although, in the present series of experiments, the ratios were calculated as described above, namely from tests between an antigen and a number of antisera in turn, it should, nevertheless, be noted that antigenic comparisons may also be

based on ratios derived from tests of one antiserum against a number of antigens in turn. In the latter instance, the ratio r_a for antiserum a against antigens A and B would then be represented

$$r_a = \frac{Ba}{Aa}.$$

The calculated r values obtained from the various tests with each of the different antigens available in the present studies are presented in Tables 1, 2 and 3, respectively. The R values calculated from these have been given in Tables 4, 5 and 6, and indicate the results obtained with antigens derived from three sources, viz. pig kidney and BHK cells or guinea-pig materials.

Table 1. *Calculated r values using microplate and tube techniques with antigens produced in IB-RS-2 cells*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
			Experiment 1			
Pak 1/54	1·00	1·00	0·84	0·93	0·30	0·28
Isr 3/63	0·23	0·22	1·00	1·00	0·28	0·27
Isr 1/57	0·16	0·16	0·67	0·81	1·00	1·00
			Experiment 2			
Pak 1/54	1·00	1·00	0·72	0·42	0·36	0·36
Isr 3/63	0·25	0·34	1·00	1·00	0·30	0·40
Isr 1/57	0·19	0·24	0·71	0·79	1·00	1·00

Table 2. *Calculated r values using microplate and tube techniques with antigens produced in BHK 21 cells*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
			Experiment 1			
Pak 1/54	1·00	1·00	0·80	0·83	0·30	0·24
Isr 3/63	0·23	0·28	1·00	1·00	0·29	0·21
Isr 1/57	0·20	0·13	0·70	0·74	1·00	1·00
			Experiment 2			
Pak 1/54	1·00	1·00	0·88	0·83	0·29	0·21
Isr 3/63	0·22	0·28	1·00	1·00	0·25	0·22
Isr 1/57	0·17	0·14	0·88	0·80	1·00	1·00

DISCUSSION

A method of comparing minor antigenic differences between strains of FMD virus of the same immunological type was developed by Bradish, Brooksby & Tsubahara (1960) and Bradish & Brooksby (1960). In that method, the product of the cross-fixation ratios of any two antigens was taken to be indicative of the degree of antigenic relationship between strains. A scheme for the taxonomic classification of FMD virus strains into subtypes was thereby shown by Davie (1964) to be applicable. When the value of the product of any two strains was $\geqslant 0{\cdot}5$ it was considered that such strains should be classified within the same subtype group and that those with values of $< 0{\cdot}5$ were different.

At the present time, a similar classification method which is feasible and currently employed is one based on the value of R, derived from the formula given. If R is $\geqslant 70\%$, then the strains under investigation are considered to fall within the same subtype group; if R is $< 70\%$, they are regarded as being of different subtypes.

Table 3. *Calculated r values using microplate and tube techniques with antigens produced in guinea-pigs*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
	Experiment 1*					
Pak 1/54	1·00	1·00	0·91	0·78	0·44	0·28
Isr 3/63	0·22	0·22	1·00	1·00	0·31	0·22
Isr 1/57	0·14	0·14	0·63	0·74	1·00	1·00
	Experiment 2*					
Pak 1/54	1·00	1·00	0·89	0·99	0·36	0·27
Isr 3/63	0·23	0·21	1·00	1·00	0·22	0·26
Isr 1/57	0·21	0·14	0·90	0·71	1·00	1·00

* Experiment 1: vesicular fluid antigen. Experiment 2: pad epithelium.

Table 4. *Calculated R values from tests using antigens produced in IB-RS-2 cells*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
	Experiment 1					
Pak 1/54	100	100	44	44	22	21
Isr 3/63	—	—	100	100	44	42
Isr 1/57	—	—	—	—	100	100
	Experiment 2					
Pak 1/54	100	100	44	43	26	29
Isr 3/63	—	—	100	100	47	38
Isr 1/57	—	—	—	—	100	100

On such criteria, the overall results obtained in the present studies with either complement-fixation method indicate that the strains Pak 1/54, Isr 3/63 and Isr 1/57 of the Asia 1 type of FMD virus are representative of different subtype groups, since the relevant R values are < 70. The reactions of the antigens in all tests were greater with the homologous antisera than with either of the respective heterologous antisera (see Figs. 1 and 2) and it was also demonstrated that each antigen was present in excess when tested with the higher dilutions of any serum.

The profiles of the histograms (see Fig. 2) in all the tests were comparable between the two techniques, which suggests that newly isolated virus strains of unknown antigenic constitution may be compared with existing subtypes using subtype-specific sera by either technique of complement fixation with equal confidence. In the absence of homologous antiserum to a newly isolated FMD virus strain, only a uni-directional test is possible. Nevertheless, a comparison of the histograms obtained with the new virus strain with those constructed from

Table 5. *Calculated R values from tests using antigens produced in BHK 21 cells*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
	Experiment 1					
Pak 1/54	100	100	43	48	24	18
Isr 3/63	—	—	100	100	45	40
Isr 1/57	—	—	—	—	100	100
	Experiment 2					
Pak 1/54	100	100	44	48	25	19
Isr 3/63	—	—	100	100	47	42
Isr 1/57	—	—	—	—	100	100

Table 6. *Calculated R values from tests using antigens produced in guinea-pig tissues*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
	Experiment 1*					
Pak 1/54	100	100	45	42	24	19
Isr 3/63	—	—	100	100	44	41
Isr 1/57	—	—	—	—	100	100
	Experiment 2*					
Pak 1/54	100	100	45	46	27	19
Isr 3/63	—	—	100	100	45	43
Isr 1/57	—	—	—	—	100	100

* Experiment 1: vesicular fluid antigen. Experiment 2: pad epithelium.

uni-directional tests using established subtype strains of virus against an identical range of subtype-specific antisera may provide some indication of the antigenic constitution of such an unknown strain, although, in the absence of reciprocal tests, the information thus made available is limited and must be interpreted with caution.

The complement-fixing activity of the same antisera used here in either technique varied between tests, not only when the antigens were produced in the same system and at different times but also with the antigens derived from the different sources. Such variability in antigen potency did not affect the general profiles of the histograms or, to some extent, the r values derived therefrom. The calculated values for R between strains, nevertheless, remained approximately constant between tests, both within a technique and in comparison between techniques (see Tables 4, 5 and 6).

In the microtechnique, the values for R (%) between strains varied by up to 4%; this was in one instance where the Pak 1/54 and Isr 1/57 antigens were derived from IB-RS-2 cells. In the tube test there was little variation except in one case (8%), again with Pak 1/54 and Isr 1/57 antigens from IB-RS-2 cells. There was a close similarity overall between the results for the two techniques; in a total of 18 values calculated for R, there was less than 5% difference in all except four instances, namely, two of 6% and one of 8% and one of 9%.

The present experiments indicate that the results obtained with the microplate method are analogous to those of the technique in tubes. The advantages of the microplate technique include the reduction in the volume of reagents required and the increased technical facility. In an analysis of subtype strains of FMD virus, the production of homologous antiserum may require 6–8 weeks and, consequently, any method which conserves such a reagent would be especially desirable. The use of the microplate method for general subtype analysis of FMD virus strains is accordingly now a regular technique in the World Reference Laboratory.

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Urban variation in infant mortality from birth injury and atelectasis in England and Wales in 1958–67

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SUMMARY

Variations in mortality attributed to birth injury and atelectasis in the 10 years 1958–67 have been investigated for each county borough of England and Wales. It became evident that diagnostic difficulties in separating these two causes must have been such that for statistical purposes they should be combined in a single rate.

Variables likely to affect the local rates such as the sex ratio of births, and rates of illegitimacy and immaturity as an ill-defined cause of death appeared to affect the mortality levels in certain towns but not sufficiently to account for the wide variations apparent in those levels. It is concluded that local surveys are necessary in the towns with highest rates to uncover differences in numbers of births in hospital, in obstetric techniques and in quality of medical care.

During the four decades from 1911 to 1951 the rate of infant mortality from all causes in England and Wales fell from 130 to 30 per 1000 live births, but in the same period the rate attributed to injury at birth and atelectasis increased from 2·7 to 6·3. Since 1951 total infant mortality has continued to fall to 18 in 1967 whilst the combined birth injury and atelectasis rate remained at its high level above six until 1961 and then declined to 4·6 in 1967, as shown in Table 1.

One of the factors responsible for this recent improvement may well have been the nation-wide investigation of peri natal mortality which was carried out by the National Birthday Trust Fund of some 25,000 babies born in March 1958 and of the 3242 neonatal deaths which occurred in England, Wales and Scotland during March, April and May of that year, reported on by Butler & Bonham (1963). Detailed inquiries concerning the deaths were made from the midwives and medical attendants at the births, and necropsies were made on most of the deaths occurring during March. This investigation must have improved the accuracy of diagnosis and registration of cause where this was obscure and it may have contributed to some of the apparent local changes and contrasts in the mortality registered during 1958–67 which are the main subject of study in the present paper.

Only the deaths of live-born infants are dealt with here, and no attempt is made to relate findings with those obtained in the 1958 survey. In the peri natal survey the coverage was not large enough to permit comparison of rates in separate

county boroughs, and the present study covers about 40 times as many deaths so as to make it possible to reveal significant differences between the towns. Many of those differences appear inexplicable but preliminary investigation of some statistically measurable factors relating to birth injury and atelectasis may throw some light on the reasons for some of the local differences, from the national rate and between individual towns.

The International Classification of Diseases contains three rubrics, No. 760, Intracranial and Spinal Injury at Birth, No. 761, Other Birth Injury, and No. 762, Atelectasis and Post-natal Asphyxia. The first two of these are combined in the Registrar General's supplementary list for separate areas during years 1958–67, so the analysis has been made for two groups of causes, 760–761 and 762. For England and Wales as a whole, however, Nos. 760 and 761 can be separated as shown in Table 1.

The table shows that after 1961 the rates for birth injury and for atelectasis both declined by 1967 by about a quarter, and the mean annual rates in 1963–7 were about 14 % lower for birth injury and 17 % lower for atelectasis than in the period 1958–62. For this reason the 10-year period 1958–67 has been divided for purposes of analysis into the two quinquennial periods, and the national standard rates used for calculating the expected deaths for the separate mortality ratios in Tables 2–4 were 2·39 and 2·04 for birth injury in the two periods, these being applied to the live-birth totals in each county borough or region in 1958–62 and 1963–7. Similarly, for atelectasis the expected deaths were obtained by applying the standard national rates of 3·41 and 2·82 to the same live-birth totals in the two periods.

Table 1. *Mortality from injury at birth (intracranial and other) and from atelectasis in England and Wales*

	No. of deaths			Rate per 1000 live births			% of 1958	
	Birth injury							
Year	C.N.S. 760	Other 761	Atelectasis 762	Birth injury 760–1	Atelectasis 762	Combined rate 760–2	Birth injury 760–1	Atelectasis 762
1921	—	—	—	1·39	1·61	3·00	57	45
1931	—	—	—	2·17	1·72	3·89	85	48
1941	—	—	—	2·55	2·18	4·73	104	61
1951	—	—	—	2·52	3·44	5·96	103	91
1958	1450	355	2647	2·44	3·57	6·01	100	100
1959	1458	355	2710	2·47	3·62	6·09	101	104
1960	1418	407	2677	2·32	3·41	5·73	95	96
1961	1508	535	2647	2·52	3·52	6·04	103	99
1962	1335	527	2675	2·22	3·19	5·41	90	99
1958–62	—	—	—	2·394	3·405	5·80	—	—
1963	1403	540	2515	2·28	2·94	5·22	93	82
1964	1397	544	2640	2·21	3·01	5·22	90	83
1965	1279	534	2333	2·10	2·70	4·80	86	76
1966	1155	468	2407	1·91	2·83	4·74	78	79
1967	1075	436	2275	1·82	2·73	4·55	75	77
1963–7	—	—	—	2·042	2·815	4·90	—	—

For the whole 10-year period the actual and expected deaths were then aggregated to obtain the overall comparative mortality ratios $100D/E$, where D were the actual and E the expected deaths. Where there is an excess over expectation the significance of this is measured by d^2/E, where d is the difference between D and E. If d^2/E exceeds 3·8 the excess is significant at the conventional level $P < 0{\cdot}05$ and is shown in the final column of Table 4; other cases where D exceeds E are indicated by 'ns'.

In Table 2 the hospital regions are examined to see whether in 1963–7 there were important differences between the rates for birth injury in the regions or between the county boroughs and other areas within each region. The regions are ranked in descending order of the C.M.R. for the region as a whole, and it is seen that those levels of mortality range from 124 and 123 in Manchester and Birmingham hospital regions to 89 and 84 in the Metropolitan and the East Anglia divisions. The four Metropolitan regions have been aggregated and the Oxford and Wessex regions combined for the purposes of Tables 2 and 3.

The C.M.R.s in the parts of the regions outside the county boroughs show some contrasts from the figures in the county boroughs, and the highest rates for such residual areas occur in the South West, Birmingham, Manchester and Welsh regions. The final column of Table 2 indicates a large excess of urban mortality in Leeds, Manchester and Liverpool hospital regions and a large urban deficiency in the South West, Oxford–Wessex and Welsh regions. Since the factors affecting the incidence of injury at birth include method of delivery, use of forceps and whether the delivery took place in a hospital, the higher mortality in the Lancashire and Yorkshire county boroughs than in residual areas of those counties is not easily explained. Why should the risk of birth injury be greater in Leeds and Bradford than in the Yorkshire dales? Are employment of women in factories or

Table 2. *Post-natal mortality from injury at birth (nos. 760–1) in 1963–7 based on numbers of live births and expressed in terms of national rate, in hospital regions, distinguishing county borough aggregates from the rest of each region*

Hospital regions ranked by C.M.R. for whole region	No. of deaths in		Comparative mortality (C.M.R.) for			Difference between C.B.S. and the rest
	County boroughs	Rest of region	Whole region	County boroughs	Rest of region	
Manchester	515	521	124	141	111	+30
Birmingham	583	612	123	125	121	+4
South western	165	432	115	92	126	−34
Leeds	381	246	112	133	85	+48
Newcastle	235	372	107	112	105	+7
Welsh	104	388	106	89	114	−25
Oxford and Wessex	93	506	98	65	96	−31
Sheffield	314	513	97	103	94	+9
Liverpool	293	130	96	107	78	+29
Metropolitan*	1507	813	89	91	87	+4
East Anglia	49	193	84	94	83	+11

* Including Greater London with the county boroughs.

larger proportions of foreign immigrants factors of importance? To those questions answers are unknown. In the South West and Wales, however, there are few large towns and in many of the country districts ready availability of specialist services for difficult births is restricted, so higher rural than urban rates are understandable. The Newcastle, Sheffield and Metropolitan regions show no important differences between the urban and rural parts.

The analysis in Table 3, unlike that in the previous table, comprises the whole 10-year period, and the C.M.R.s for birth injury and also for atelectasis in the county borough aggregates within each region are shown for each of the 5-year periods and the whole period. The regions are ranked in descending order of the overall ratios for birth injury only, which ranged from 133 in Manchester and 124 in Liverpool and Birmingham regions to below 85 in the Oxford and Wessex regions and East Anglia. The north-west to south-east downward trend of rates characteristic of mortality from so many causes was very evident in both periods for birth injury. Atelectasis rates in 1958–62, however, were highest in Wales, followed by Liverpool and the Oxford–Wessex area, but in 1963–7 they were high in the Manchester and Leeds regions. In addition to showing the facts for both causes of death, a purpose of the table is to see whether the changes in registered mortality occurring in the second 5-year period indicated in any region a transfer of diagnosis from one cause to the other. There was a transfer to atelectasis in Manchester and Newcastle regions and one of the opposite kind, from atelectasis to birth injury, was indicated in Wales and the Oxford and Wessex area.

The most remarkable change occurring during the 10 years was the decline in Liverpool mortality from both causes (141 to 107 for birth injury and 139 to

Table 3. *Comparative post-natal mortality from birth injury and atelectasis based on numbers of live births in* 1958–62 *and* 1963–7 *in the county boroughs of each hospital region*

Hospital regions in which county boroughs are located	Birth injury (760–761)					Atelectasis (762)				
	Total deaths 1958–67	C.M.R. in 1958–62	C.M.R. in 1963–7	C.M.R. in 1958–7	C.M.R. change in 1963–7	Total deaths 1958–67	C.M.R. in 1958–62	C.M.R. in 1963–7	C.M.R. in 1958–7	C.M.R. change in 1963–7
Manchester	1028	125	141	133	+16	1377	109	140	129	+31
Liverpool	717	141	107	124	−34	1005	139	101	121	−38
Birmingham	1218	123	125	124	+2	1387	103	101	102	−2
Leeds	723	105	133	120	+25	1017	108	126	117	+18
Newcastle	540	118	112	115	−6	665	96	108	101	+12
Sheffield	658	102	103	103	+1	970	107	102	104	−5
Metropolitan*	3008	91	100	94	+9	4588	100	106	102	+6
Welsh	247	94	89	92	−5	445	162	98	132	−64
South western	342	84	92	90	+8	476	85	99	92	+14
East Anglia	88	70	94	82	+24	88	53	69	60	+16
Oxford and Wessex	224	86	65	75	−21	472	129	95	112	−34

* The C.M.R.s are for Greater London conurbation: the total deaths include 97 from birth injury and 179 from atelectasis in the five county boroughs of the region which were outside Greater London.

Table 4. *Comparative mortality based on numbers of live births and attributed to birth injury and atelectasis in each county borough of England and Wales*

County boroughs alphabetically in each hospital region	Birth injury (760, 761)					Atelectasis (762)				
	Total deaths 1958–67	C.M.R.s (per 100 expected) 1958–62	1963–7	1958–67	Significant excess (d^2/E)*	Total deaths 1958–67	C.M.R.s (per 100 expected) 1958–62	1963–7	1958–67	Significant excess (d^2/E)*
Manchester region										
Barrow in Furness	18	85	47	65	—	44	82	173	128	ns
Blackburn	24	59	65	62	—	71	121	141	130	5
Blackpool	49	123	112	117	ns	75	121	130	126	4
Bolton	66	80	145	111	ns	145	174	182	177	8
Burnley	48	169	166	167	13	65	159	163	161	15
Bury	21	81	83	82	—	44	136	110	123	ns
Manchester C.B.	420	147	154	149	68	466	109	129	118	13
Oldham	128	190	391	287	16	88	180	96	140	12
Preston	48	97	116	104	ns	74	125	101	114	ns
Rochdale	42	95	157	125	ns	58	106	188	140	6
Salford	87	114	151	131	6	126	90	181	133	11
Stockport	48	140	49	93	—	95	129	133	131	4
Wigan	29	124	73	100	—	26	50	79	61	—
Total C.B.s	1028	125	141	133	—	1377	109	140	129	—
Liverpool region										
Birkenhead	96	146	171	156	19	83	143	78	116	ns
Bootle	42	121	81	104	ns	60	128	63	106	ns
Chester	36	143	157	109	6	24	61	82	71	—
Liverpool C.B.	384	151	93	123	18	594	148	95	125	30
St Helens	34	87	77	82	—	94	145	181	159	21
Southport	23	111	79	96	—	21	45	82	65	—
Warrington	41	157	110	134	4	55	101	158	129	4
Wallasey	61	122	142	132	5	74	133	122	124	4
Total C.B.s	717	141	107	124	—	1005	137	101	127	—
Birmingham region										
Birmingham C.B.	588	123	123	125	27	752	112	115	117	10
Burton-on-Trent	21	99	99	99	—	31	114	93	106	ns
Coventry	253	128	245	176	83	229	65	69	67	—
Dudley †	33†	142	116‡	128†	ns	35†	117	79‡	97†	—
Smethwick†	21†	111	44‡	77†	—	23†	83	37†	60†	—
Stoke-on-Trent	60	87	33	62	—	168	123	122	123	7
Walsall	70	130	154	141	8	79	111	95	103	ns
West Bromwich†	41†	73	128‡	104†	ns	46†	117	103‡	111†	ns
Wolver-hampton†	107†	207	121‡	163‡	15	102†	97	128‡	113†	ns
Worcester	24	77	115	95	—	29	65	100	90	—
Total C.B.s	1218	123	125	124	—	1494	103	101	102	—

Table 4 (*cont.*)

County boroughs alphabetically in each hospital region	Birth injury (760,761) Total deaths 1958–67	C.M.R.s (per 100 expected) 1958–62	1963–7	1958–67	Significant excess (d^2/E) *	Atelectasis (762) Total deaths 1958–67	C.M.R.s (per 100 expected) 1958–62	1963–7	1958–67	Significant excess (d^2/E)*
Leeds region										
Bradford	161	144	115	131	12	302	149	204	174	97
Dewsbury	24	107	128	117	ns	29	101	100	100	—·
Halifax	31	69	104	86	—	43	71	100	84	—
Huddersfield	27	56	48	52	—	75	109	86	103	ns
Kingston upon Hull	109	75	96	86	—	198	126	97	109	ns
Leeds C.B.	301	119	185	150	51	299	94	121	106	ns
Wakefield	21	82	124	101	ns	45	197	104	155	8
York	49	112	146	129	ns	26	20	77	43	—
Total C.B.s	723	105	133	120	—	1017	108	126	117	—
Newcastle region										
Carlisle	41	190	83	143	5	27	67	66	66	—
Darlington	54	175	169	172	16	34	67	88	77	—
Gateshead	36	81	88	83	—	40	82	45	66	—
Middlesbrough	74	56	107	97	—	85	66	102	81	—
Newcastle upon Tyne C.B.	107	112	89	102	ns	115	73	86	78	—
South Shields	67	149	153	151	11	85	85	92	88	—
Sunderland	99	113	145	125	5	201	178	192	184	16
Tynemouth	31	109	114	111	ns	51	124	146	134	4
West Hartlepool	31§	61	80‖	80§	—	57§	118	106‖	113§	ns
Total C.B.s	540	118	112	115	—	695	96	108	101	—
Sheffield region										
Barnsley	24	73	98	84	—	68	172	163	167	11
Derby	61	119	123	124	4	92	150	113	132	4
Doncaster	31	92	99	93	—	59	116	139	132	5
Grimsby	24	52	65	58	—	55	83	101	91	—
Leicester	125	126	103	116	ns	151	92	85	88	—
Lincoln	24	57	72	79	—	36	93	71	87	—
Nottingham	111	78	139	107	ns	229	163	78	128	10
Rothernam	61	182	164	163	15	65	126	125	124	ns
Sheffield C.B.	167	115	65	91	—	215	56	115	87	—
Total C.B.s	628	102	103	103	—	970	107	102	104	—
Metropolitan regions										
Brighton	33	62	67	64	—	61	89	79	84	—
Canterbury	6	54	58	56	—	12	57	96	75	—
Eastbourne	9	57	68	63	—	20	115	69	93	—
Hastings	20	96	106	66	—	16	67	54	61	—
Southend-on-Sea	29	75	31	56	—	70	92	97	94	
Greater London	2911	91	106	94	—	4409	100	106	102	—
Total C.B.s plus Greater London	3008	—	—	—	—	4588	—	—	—	—

Table 4 (*cont.*)

County boroughs alphabetically in each hospital region	Birth injury (760, 761)					Atelectasis (762)				
	Total deaths 1958–67	C.M.R.s (per 100 expected) 1958–62	1963–7	1958–67	Significant excess (d^2/E) *	Total deaths 1958–67	C.M.R.s (per 100 expected) 1958–62	1963–7	1958–67	Significant excess (d^2/E)*
East Anglia region										
Ipswich	41	70	99	83	—	45	60	83	70	—
Great Yarmouth	11	65	62	65	—	13	47	63	55	—
Norwich	36	72	104	87	—	30	48	57	52	—
Total C.B.s	88	70	94	82	—	88	53	69	60	—
Oxford and Wessex regions										
Northampton	39	82	91	87	—	72	139	92	115	ns
Oxford C.B.	31	94	68	81	—	50	90	102	96	—
Portsmouth	42	41	63	51	—	139	138	96	115	ns
Reading	45	137	44	90	—	80	143	80	114	ns
Southampton	67	102	58	82	—	131	123	102	113	ns
Total C.B.s	224	86	64	75	—	472	129	95	112	—
South Western region										
Bath	42	168	131	151	7	39	85	117	100	—
Bournemouth	37	130	62	96	—	37	39	97	69	—
Bristol	169	80	130	105	ns	234	97	115	105	ns
Exeter	13	35	57	46	—	54	159	114	137	5
Gloucester	17	64	44	54	—	31	58	82	70	—
Plymouth	54	81	47	64	—	81	68	69	68	—
Total C.B.s	332	84	92	90	—	476	85	99	92	—
Welsh region										
Cardiff	115	106	102	104	ns	222	183	100	144	8
Merthyr Tydfil	21	127	57	94	—	70	294	151	223	47
Newport	45	111	65	86	—	84	158	77	114	ns
Swansea	66	109	103	105	ns	69	84	96	86	—
Total C.B.s	247	110	81	100	—	445	162	98	132	—

* Excess over expectation but not statistically significant ($P > 0{\cdot}05$) is denoted by 'ns', the value of d^2/E being smaller than 3·8.

† Deaths and rates are for 7 years 1958–64.

‡ Ratios for 1963–4.

§ Deaths and rates are for 9 years 1958–66.

‖ Ratios for 1962–6.

101 for atelectasis) contrasted with increases in the Manchester region (125 to 141 and 109 to 140).

Mortality in separate county boroughs

Table 4 shows the number of post-natal deaths in the 10 years 1958–67 from birth injury (intracranial and other) and from atelectasis in each of the county boroughs. The total deaths in all county boroughs and Greater London combined attributed to birth injury were 8653, and the total attributed to atelectasis and post-natal asphyxia were 12,627. The county boroughs are arranged according to the hospital region in which they are located, and in alphabetical order within each region. The towns where the total number of deaths exceeded the number expected (if the national rates in Table 1 within the appropriate 5-year period had been applied to the total number of live births in each instance) are indicated in the final column by the values of d^2/E if the excess was statistically significant or by the letters ns where that figure was below the critical value of 3·8 (corresponding to probability $P < 0{\cdot}05$).

The towns which showed a statistically significant excess of actual over expected deaths at the 5% level of probability ($P < 0{\cdot}05$ for $d^2/E = 3{\cdot}8$) for the whole period 1958–67 for both causes of death are listed in Table 5 in their regional groups, and also those where there was significant excess for one of the causes combined with an insignificant excess (ns) for the other cause.

There were 20 towns thus identified as having noteworthy excessive mortality from birth injury and atelectasis, and eleven of these were clustered round Manchester and Liverpool, and another seven were in the Newcastle, Leeds and Sheffield regions, leaving one in the Midlands, one in Wales and none in the southern half of England. After each town is shown in parentheses the mean annual combined death rate from the two causes in 1958–67, and in every case

Table 5. *County boroughs with notably high mortality in Table* 4

Hospital region	A	B	C
Manchester	Burnley (8·6)*	—	Blackpool (6·5)
	Manchester (7·0)	—	Bolton (7·4)
	Oldham (11·5)	—	Rochdale (6·1)
	Salford (6·1)	—	—
Liverpool	Liverpool (6·3)	Birkenhead (7·3)	—
	Warrington (6·7)	—	—
	Wallasey (7·2)	—	—
Birmingham	Birmingham (6·3)	—	—
Leeds	Bradford (8·3)	Leeds (6·6)	—
Newcastle	Sunderland (8·8)	—	Tynemouth (6·6)
Sheffield	Derby (6·9)	Rotherham (6·6)	Nottingham (6·1)
Welsh	—	—	Cardiff (6·8)

A. C.B.s with statistically significant excess of actual over expected mortality from both birth injury and atelectasis.

B. C.B.s with significant excess for birth injury and insignificant excess from atelectasis.

C. C.B.s with significant excess for atelectasis and insignificant excess for birth injury.

* Figures in parentheses show the mean annual combined death rates from the two causes in 1958–67.

this figure exceeded six per 1000 live births. In addition there were 9 other towns with rates of six or more but which failed to satisfy the criteria of statistical significance of groups A, B or C, namely: Preston (6·0), Stockport (6·1), St Helens (6·9), Wakefield (6·8), Barnsley (7·1), Doncaster (6·1), Coventry (6·1), Walsall (6·1), Bath (6·4).

Apart from the disturbances arising from differential diagnosis of the cause of death, factors likely to affect the comparisons between towns are differences in sex ratio of births and in rates of illegitimacy and immaturity and these will be examined below. Although many environmental factors can be cited to account for the northern excess of infant mortality generally, it is not clear why these should affect deaths arising from injury at birth.

Sex ratio variations

Since the risk of birth injury is greater for male than for female infants this might be a factor in some of the differences between death rates in large towns. In England and Wales during 1958–67 there were 4,218,000 live births of males and, 3,983,500 of females, giving a sex ratio of 1·06. The rates per 1000 live births are shown in Table 6. The sex ratio between numbers of deaths was greatest for brain injuries (1·71) as might be expected since the size of head tends to be larger in male infants; but the male excess was also pronounced (1·54) for other birth injuries, and for all combined it was 1·66. For atelectasis and post-natal asphyxia the ratio was 1·42. The two birth injury groups are not distinguished in the statistics for separate county boroughs and they have to be aggregated for the comparisons which follow. The expected deaths of males from birth injury in a county borough would be 2·70 times the total live births of males in the 10 years divided by 10,000, and the expected deaths of females would be 1·73 times the total live births of females divided by 10,000. Among the 20 towns with specially high death rates for the combined sexes, in some the high rate might be enhanced by an unusual excess of males born alive and in others by an unusually large excess of the male over the female death rate.

Table 7 shows the various sex ratios (male/female) and the numbers of deaths and rates per 1000 live births for each sex in 1958–67 in five groups of county boroughs where mortality from birth injury for the combined sexes was high compared with Greater London and the country as a whole. The variations in sex ratio among the births did not exceed 3% and are less important than the differences between sex ratios shown by the death rates. The five urban groups all had high death rates among male infants, exceeding 3·6 per 1000 compared with the national figure of 2·70 and the Greater London rate of 2·60, but the last two

Table 6. *Deaths from birth injury and atelectasis*, 1958–67, *according to sex*

	No. of deaths		Sex ratio	Rate/1000	
	M	F	(deaths)	M	F
Intracranial injury (760)	8,508	4,971	1·71	2·70	1.73
Other birth injury (761)	2,870	1,869	1·54 } 1.66		
Atelectasis (762)	15,348	10,778	1.42	3·64	2·71

columns show that the excess in female rates (compared with 1·73) was relatively less than in males, though not appreciably so in Manchester area. Testing the rates for each sex for significance of the excess, in Manchester area the actual deaths exceeded the numbers expected by highly significant amounts (d^2/E being 107 for males and 53 for females) so the cause of this operated for each sex alike.

In Liverpool area, however, the excess for males, 367 actual compared with 271 expected, was highly significant ($d^2/E = 34$) but for females the excess, 181 compared with 161 expected, was inconsiderable. In Bradford the expected deaths were 77 males and 45 females, the excess of males being statistically significant ($d^2/E = 9{\cdot}5$) but for females it was below the conventional level (d^2/E less than 3·8). In Rotherham the expected deaths of males were 22 and the excess was significant ($d^2/E = 17$) whilst for females the actual and expected deaths were 20 and 14 giving an insignificant excess. In Derby the expected deaths of males were 32 and the excess was significant ($d^2/E = 6{\cdot}1$) but for females there was no excess.

It is apparent that the multiple factors responsible for mortality excess from birth injury in certain towns affect each sex but tend to enhance the risk for male infants more than that for females. The much stronger tendency for males to be more affected than females in the Liverpool area than was the case in the Manchester area is connected with a curious contrast between the trends in the proportions of atelectasis and birth injury in the two areas, as will be seen in the next section.

Atelectasis and post-natal asphyxia

In the absence of an autopsy a differential diagnosis of cause of death between birth injury and atelectasis is often difficult and the choice of which to write on the death certificate may be affected by personal bias, obstetric experience and perhaps by teaching at the medical school in the neighbourhood. Indications of

Table 7. *Sex ratios for birth injury in urban areas with a specially high mortality from that cause*, 1958–67

Urban group	Sex ratio between: Live births	Deaths M/F	Rates M/F	Numbers of deaths in 1958–67: M	F	Rate/1000 live births: M	F	% of national: M	F
Manchester, Salford, Oldham, Rochdale	1·05	1·74	1·63	433	265	4·52	2·82	167	163
Liverpool, Birkenhead, Wallasey	1·08	2·03	1·89	367	181	3·66	1·94	136	112
Bradford	1·09	1·82	1·68	104	57	3·65	2·17	135	125
Rotherham	1·08	2·55	1·98	41	20	5·06	2·55	187	146
Derby	1·07	2·42	2·24	46	19	3·93	1·75	146	101
Greater London	1·06	1·58	1·50	1,788	1,129	2·60	1·73	97	100
England and Wales	1·06	1·63	1·56	11,369	6,993	2·70	1·73	100	100

such effects are seen when the death rates in Manchester and Liverpool areas are compared in the periods 1958–62 and 1963–7. There appears to have been a change in fashion in the Liverpool area causing transfer of deaths from atelectasis to birth injury during the 10-year period, as shown by the rates and numbers of deaths in Table 8 in the Manchester and Liverpool groups of county boroughs.

In England and Wales as a whole atelectasis rates declined between 1958–62 and 1963–7 by 23 % among males and by 15 % among females. In the Manchester area the rates fell by only 4 % in males and 7 % in females, but in the Liverpool area the rates fell sharply by 47 % in males and by 54 % in females. The national rates for birth injury also declined, by 16 % in males and 10 % in females, slighter falls than occurred for atelectasis. In the Manchester area, however, the birth injury rates increased by 15 % for each sex, suggesting some transfer from atelectasis as the certified cause. In Liverpool area on the other hand the birth injury rate fell by 28 % in males but by only 5 % in females in strong contrast with the 54 % fall for atelectasis in that sex. The very large drop in the 5-year total of deaths attributed to atelectasis between 1958–62 and 1963–7, from 497 to 231, must have been due to a progressive change in diagnostic fashion in that area causing many such deaths to be classified to other causes but not to birth injury.

In Table 4 the changes in rate of dying from birth injury or from atelectasis between 1958–62 and 1963–7 are indicated approximately by the differences between the C.M.R.s for those periods. Thus for Bolton the C.M.R. for birth injury was 80 in the first period and 145 in the second and the difference of + 65 is a rough index of the change in mortality which occurred during the 10 years (the mean annual change being one fifth of this). For atelectasis in the same town the C.M.R. showed no appreciably change (174 to 182). Apart from the Manchester group of boroughs referred to above, the towns showing *increases* of 10 or more in the C.M.R.s for *both* causes were: Halifax, Leeds, York, Chester, Middlesbrough,

Table 8. *Atelectasis and birth injury rates in Manchester (with Salford, Oldham and Rochdale) and Liverpool (with Birkenhead and Wallasey)*

		Manchester area		Liverpool area		England and Wales	
		1958–62	1963–7	1958–62	1963–7	1958–62	1963–7
Number of deaths							
Atelectasis	M	232	220	293	144	8058	7290
	F	144	136	204	87	5298	4880
Birth injury	M	207	226	221	146	5938	5437
	F	122	143	96	85	3446	3394
Rate/1000 live births							
Atelectasis	M	4·71	4·51	5·50	2·94	4·29	3·32
	F	3·09	2·87	4·10	1·88	2·78	2·35
Birth injury	M	4·21	4·82	4·15	2·98	2·94	2·48
	F	2·62	3·02	1·93	1·83	1·81	1·63
Rate % of previous period							
Atelectasis	M	—	96	—	53	—	77
	F	—	93	—	46	—	85
Birth injury	M	—	115	—	72	—	84
	F	—	115	—	95	—	90

Grimsby, Bristol and Worcester. No reason can be suggested here for the upward trends for both causes in these towns, but they are not likely to have arisen from mere changes in fashion of diagnosis on death certificates. In contrast there were six towns showing decreases in the C.M.R. between 1958–62 and 1963–7 amounting to 10 or more for each of the causes, viz. Bootle, Liverpool, Dudley, Smethwick, Southampton and Newport.

Table 9. *Combined rates of mortality per* 1000 *live births from birth injury and atelectasis in* 78 *county boroughs during* 1958–67 *with separate indices for each cause in* 1938–62 *and* 1963–7. *Rates of illegitimacy and of mortality attributed to immaturity per* 1000 *live births in* 1963–7

	Rank	Combined rate: Mean annual 1958–67	Combined rate: Change in 5 years	Birth injury only: 1958–62	Birth injury only: 1963–7	Atelectasis only: 1958–62	Atelectasis only: 1963–7	Illegitimacy 1963–7	Immaturity 1963–7	Number of live births in: 1958–62	Number of live births in: 1963–7
Oldham	1	11·47	−1·46	4·60	8·04	6·14	4·16	124	1·75	1,954	2,063
Sunderland	2	8·83	+0·18	2·68	2·98	6·06	5·94	63	3·42	3,860	3,629
Burnley	3	8·60	−1,12	4·02	3·40	5·14	4·64	104	2·32	1,291	1,292
Bradford	4	8·33	−0·34	3·46	2·36	5·04	5·80	111	3·07	5,381	5,729
Bolton	5	7·47	−1·24	1·88	2·98	4·82	5·16	89	2·02	2,617	2,754
Birkenhead	6	7·34	−2·72	4·70	3·64	4·00	2·34	75	4·22	2,611	2,800
Wallasey	7	7·15	+0·12	2·40	3·68	4·96	3·46	76	4·03	1,858	1,845
Barnsley	8	7·11	−0·98	1·76	2·14	5·84	4·58	73	2·70	1,374	1,182
Manchester	9	7·03	−0·45	3·52	3·16	3·72	3·66	141	3·49	13,506	12,467
Wakefield	10	6·96	−3·17	1·81	2·54	6·74	2·84	78	2·97	920	942
Derby	11	6·89	−2·10	2·84	2·12	5·10	3·22	115	0·76	2,116	2,364
St Helens	12	6·88	−0·28	2·08	1·54	4·94	5·20	58	4·43	1,916	1,806
Cardiff	13	6·85	−3·82	2·54	2·10	6·22	2·84	89	2·95	4,787	5,150
Warrington	14	6·75	+0·06	3·46	2·26	3·26	4·52	83	3·81	1,327	1,419
Tynemouth	15	6·65	−0·68	2·80	2·24	4·12	4·14	76	4·85	1,275	1,156
Wolverhampton	16	6·64	—	4·96	2·42†	3·52	2·38†	119	2·87†	2,665	3,184†
Leeds	17	6·64	+1·20	2·84	3·82	3·20	3·42	104	2·36	8,797	9,216
Rotherham	18	6·57	+0·02	3·88	3·38	2·28	3·60	61	0·76	1,545	1,832
Blackpool	19	6·49	−0·96	2·84	2·30	4·14	3·70	124	1·35	1,837	2,000
Bath	20	6·44	−0·88	4·00	2·68	2·88	3·32	84	1·74	1,246	1,266
Newport	21	6·37	−3·30	2·64	1·80	5·38	2·92	78	3·47	1,967	2,129
Birmingham	22	6·30	−·104	2·94	2·52	3·88	3·26	112	2·40	20,819	21,841
Liverpool	23	6·28	−3·36	2·72	1·90	5·24	2·70	85	3·67	15,850	14,372
Rochdale	24	6·15	+1·92	1·96	3·22	3·18	3·94	108	2·72	1,435	1,618
Salford	25	6·14	+0·56	2·72	3·10	3·14	3·32	128	4·46	3,005	2,960
Nottingham	26	6·18	−2·66	1·86	2·52	5·58	2·26	143	4·44	5,806	6,077
Coventry	27	6·10	−1·72	3·68	4·28	2·28	1·96	81	2·82	5,886	6,535
Stockport	28	6·09	−3·38	3·38	1·06	4·40	3·34	70	6·85	2,089	2,586
Walsall	29	6·08	−1·62	3·06	2·60	3·84	2·68	64	4·64	2,188	2,760
Doncaster	30	6·06	+0·02	2·10	2·16	3·90	3·96	99	6·42	1,525	1,464
Preston	31	6·00	−1·28	2·32	2·38	4·32	2·98	96	3·57	2,065	2,014
South Shields	32	5·97	−0·42	3·28	3·14	2·90	2·60	70	2·94	2,069	1,909
Dudley	33	5·84*	—	3·38	2·38†	3·68	2·24†	65†	3·24†	1,006	1,038†
Darlington	34	5·77	−1·42	4·18	2·66	2·30	2·40	81	2·66	1,389	1,435
Merthyr Tydfil	35	5·76	+3·54	3·04	0·98	1·46	6·06	52	5·47	958	1,024

Table 9 (*cont.*)

	Rank	Combined rate: Mean annual 1958–67	Combined rate: Change in 5 years	Birth injury only: 1958–62	Birth injury only: 1963–7	Atelectasis only: 1958–62	Atelectasis only: 1963–7	Illegitimacy 1963–7	Immaturity 1963–7	Number of live births in: 1958–62	Number of live births in: 1963–7
Bury	36	5·72	−1·74	1·94	1·72	4·66	3·12	81	3.29	1,030	1,278
Dewsbury	37	5·71	−0·50	2·56	2·62	3·40	2·84	85	5·91	939	913
Bristol	38	5·70	+0·72	1·92	2·80	3·42	3·46	92	1·47	6,987	7,298
Burton-on-Trent	39	5·66	−1·22	2·38	2·20	3·88	2·56	163	2·04	907	986
Leicester	40	5·64	−0·92	3·08	2·12	3·02	3·06	111	4·84	5,057	5,086
Sheffield	41	5·62	+0·82	2·74	3·34	1·90	3·28	77	2·50	8,000	8,490
Barrow in Furness	42	5·60	+1.56	2·04	1·24	2·80	5·12	73	3·16	1,076	1,138
Chester	43	5·53	+0·06	3·42	3·22	2·08	2·34	83	1·79	1,055	1,113
West Hartlepool	44	5·44	−2·36	2·58	1·26	4·04	3·00	72	1·91	1,630	1,593
Blackburn	45	5·43	−0·18	1·40	1·34	4·12	4·00	86	3·56	1,702	1,796
Bootle	46	5·40	−3·88	2·82	1·64	4·54	1·80	56	4·55	1,943	1,672
Exeter	47	5·38	−1·96	0·94	1·18	5·42	3·22	84	1·03	1,180	1,361
Southampton	48	5·37	−2·54	2·44	1·20	4·20	2·90	105	1·63	3,616	3,801
Kingston upon Hull	49	5·33	−1·26	1·98	1·98	4·08	2·62	97	2·86	5,830	5,750
Stoke-on-Trent	50	5·25	−1·18	2·10	0·68	4·24	3·48	63	2·25	4,302	4,439
Eastbourne	51	5·25	+0·14	1·22	1·38	3·96	3·94	100	2·50	657	720
Carlisle	52	5·17	−3·22	4·52	1·70	2·26	1·86	79	3·80	1,326	1,288
Swansea	53	4·97	−0·58	2·60	2·12	2·16	2·06	60	4·16	2,768	2,828
Northampton	54	4·97	−1·98	1·84	1·86	4·62	2·62	83	1·53	1,731	2,358
Portsmouth	55	4·92	−1·43	0·98	1·30	4·70	2·86	140	2·54	3,905	3,707
Huddersfield	56	4·85	+2·40	1·34	1·98	3·64	2·74	121	6·53	2,219	2,451
Newcastle upon Tyne	57	4·74	−0·92	2·63	1·84	2·52	2·44	102	4·24	4,985	4,346
Oxford	58	4·72	−0·84	2·26	1·40	2·58	2·90	111	1·94	1,574	1,860
Middlesbrough	59	4·71	+0·84	2·20	2·20	2·24	2·88	92	5·24	3,538	3,170
Worcester	60	4·64	+1·24	1·82	2·38	2·20	2·88	61	4·07	1,092	1,180
Halifax	61	4·51	+0·90	1·64	2·12	2·42	2·84	111	4·49	1,575	1,693
Lincoln	62	4·35	−0·36	1·36	2·16	3·16	2·02	86	2·91	1,328	1,370
Hastings	63	4·27	−1·06	2·52	2·20	2·28	1·54	117	1·33	790	911
Bournemouth	64	4·25	−0·42	3·10	1·28	1·36	2·76	128	4·13	1,617	1,888
Wigan	65	4·22	−0·96	2·98	1·50	1·74	2·22	48	4·79	1,278	1,335
Grimsby	66	4·16	+0·04	1·24	1·28	2·90	2·90	102	4·39	1,926	1,867
Southport	67	4·06	−0·22	2·66	1·62	1·52	2·34	42	2·94	1,050	1,111
Brighton	68	4·03	−1·87	3·42	1·80	3·02	2·22	131	2·48	2,330	2,444
Reading	69	3·90	−1·97	3·26	0·81	1·62	2·10	92	4·27	2,092	2,443
Gateshead	70	3·89	−1·62	1·90	1·80	2·80	1·28	62	3·88	2,000	1,884
Ipswich	71	3·85	+0·30	1·66	2·00	2·04	2·00	72	2·31	2,053	2,405
York	72	3·77	+2·86	1·52	3·00	0·82	2·20	92	5·09	1,716	1,729
Canterbury	73	3·67	+0·50	1·28	1·18	2·14	2·74	79	2·74	471	511
Southend-on-Sea	74	3·65	−0·51	1·83	0·74	2·22	2·82	100	4·82	2,232	2,408
Norwich	75	3·49	+0·50	1·62	2·04	1·62	1·60	94	2·47	1,047	1,864
Gloucester	76	3·34	+0·72	1·52	0·88	1·96	2·32	106	3·10	1,315	1,547
Plymouth	77	3·20	+0·56	1·26	0·98	2·22	1·94	82	2·77	3,698	3,896
Great Yarmouth	78	3·15	−0·18	1·62	1·28	1·62	1·78	117	7·41	737	783
England and Wales		5·35	−0·90	2·39	2·06	3·41	2·89	76	3·02	—	—

Towns which ceased to be county boroughs before 1963 have been omitted as also when boundary changes during 1963–7 interfered with the rate computation and correction was not possible.

* Annual means for 1958–65. † Annual means for 1963–5.

In 37 of the towns, however, the changes in mortality with time during 1958–67 for birth injury and atelectasis showed no correspondence in sign (i.e. up or down) and in some instances such as Nottingham and Wakefield the opposing changes were large in amount. The conclusion must be that *uncertainty as to which cause to enter on the death certificate is often such that no reliance can be placed on the separate figures resulting, and it is more realistic to combine the statistics for categories 760–2 as a single cause group* and observe whether the combined rate rose or fell during the 10 years. For this purpose the combined death rates attributed to birth injury and atelectasis in the two 5-year periods are shown in Table 9. In England and Wales the combined rate was 5·80 per 1000 live births in the first period and 4·90 in the second, and 5·35 in the whole 10 years. The table shows also the difference between the combined rates in the two periods, and the 25 towns where that difference was positive (including five where the difference was less than 0·1), indicating a rising trend during the 10 years, are seen to be:

(*a*) Showing increases over: 1·0 Merthyr Tydfil (3·5), York (2·9), Huddersfield (2·4), Rochdale (1·9), Barrow-in-Furness (1·6), Worcester (1·2) and Leeds (1·2).

(*b*) Showing increases of 0·1 to 0·9: Halifax, Middlesbrough, Sheffield, Bristol, Gloucester, Plymouth, Salford, Canterbury, Norwich, Ipswich, Sunderland, Eastbourne and Wallasey.

Of these 20 towns where mortality rose appreciably during the 10 years, five were in the West Riding of Yorkshire and five in Lancashire and Cheshire, and another group of adjacent towns were Bristol, Gloucester and Worcester. Since in England and Wales as a whole the death rate from the combined causes in 1963–7 was *less* than in 1958–62 by 0·9 per 1000 live births, the reasons for the increases in the towns named above seem to need investigation by the Health Departments concerned.

By way of contrast, substantial improvements of 2·0 or more occurred in the combined rate in the following county boroughs: Bootle, Liverpool, Stockport, Cardiff, Newport, Carlisle, Wakefield, Birkenhead, Nottingham, Southport, West Harlepool and Derby.

Frequency of illegitimacy and of deaths from immaturity without other cause

Table 9 shows that the illegitimacy rate per 1000 live births during 1958–67 ranged from 163 in Burton-on-Trent to 42 in Southport, and it is conceivable that towns with a high rate of illegitimacy may tend to have a higher risk of birth injury and atelectasis. This can be tested by comparing the median illegitimacy rates in successive groups of 10 towns in the table, where they are ranked in descending order of the combined mortality rate, and by looking at the towns with highest illegitimacy rates in detail.

In successive groups of 20, 20, 20, 18 towns in the order of the table the median illegitimacy rates were 85, 87, 88, 95, showing no positive relationship with the combined birth injury and atelectasis death rates, but rather the reverse. However, the 10 towns with illegitimacy rates over 120 were as shown in Table 10 and their median death rate from birth injury and atelectasis was 5·90, slightly above the

median 5·68 for all county boroughs. In five of those towns the death rate was high, namely the Manchester, Salford and Oldham group and also Blackpool and Nottingham. The average illegitimacy rate in England and Wales was 76 per 1000 live births and any rates of 100 or more may be considered abnormally high. There were 18 such towns in addition to those listed in Table 10 (with rates over 120), and 9 of them were textile or Midland industrial towns, viz. Wolverhampton (119), Derby (115), Birmingham (112), Bradford, Halifax and Leicester (111), Rochdale (108), Burnley and Leeds (104). All of these except Halifax had combined birth injury and atelectasis rates much above average (median 6·64). The others were seven coastal towns, Great Yarmouth and Hastings (117), Southampton (105), Grimsby and Newcastle (102), Eastbourne and Southend (100), together with Oxford (111), and Gloucester (106), with median combined mortality 4·27. In the coastal towns high prevalence of illegitimacy is common, but no association with an enhanced mortality from birth injury seems to result.

Immaturity without mention of a more definite cause of death peculiar to early infancy (No. 776 of the International Classification) accounted in the years 1963–7 for mean annual rates ranging from 0·76 per 1000 live births in Derby and Rotherham to 7·41 in Great Yarmouth. The correlation between these rates and death rates from birth injury and atelectasis was −0·37, and the negative association arises almost entirely from two towns at the extremes of Table 9. Oldham recorded an abnormally high rate from birth injury in 1963–7, namely 8·04 with no other town exceeding four, and its immaturity rate of 1·75 was low compared with the national rate of 3·02. It seems probable that a considerable number of deaths of immature infants were classified to birth injury as most likely cause in the absence of definite indications of any other reason for the death. If so that would help to account for the position of Oldham at the top of the table with a figure of 11·47 for the combined rate with atelectasis in the whole period.

At the other end of the table Great Yarmouth recorded the highest rate for immaturity (7·41) and the lowest rate for combined birth injury and atelectasis

Table 10. *County boroughs with illegitimacy frequencies exceeding* 120 *per* 1000 *live births, with corresponding immaturity rates, in* 1963–7

	Illegitimacy rate 1963–7	Death rate attributed to immaturity 1963–7	Death rate from birth injury and atelectasis 1958–67
County borough			
Burton-on-Trent	163	2·04	5·66
Nottingham	143	4·44	6·18
Manchester	141	3·49	7·03
Portsmouth	140	2·54	4·92
Brighton	131	2·48	4·03
Bournemouth	128	4·13	4·25
Salford	128	4·46	6·14
Oldham	124	1·75	11·47
Blackpool	124	1·35	6·49
Huddersfield	121	6·53	4·85
Median	130	3·02	5·90

which suggests that some deaths of immature infants with uncertain evidence of either of those causes may have been classed to the ill-defined category of immaturity. There is no significant evidence of an association in the other 76 towns though transfers either way between Nos. 760–62 and No. 766 no doubt occurred in some of them.

Although the variables illegitimacy frequency, deaths from immaturity without other known cause and unusual excess of male infants affect some of the combined rates of death from birth injury and atelectasis, most of the differences between county boroughs, ranging from three to eight per 1000 live births as shown by the third column of Table 9, must arise from local variations in the number of births in hospital, obstetric techniques and quality of medical care of births. These cannot be evaluated statistically without local surveys on the lines of the investigation of Perinatal Mortality which was carried out in 1958 under the auspices of the National Birthday Trust (Butler & Bonham, 1963). Such surveys seem to be desirable, particularly in the first 18 towns in Table 9 where the mortality rate in 1958–67 was 6·5 or more per 1000 live births annually.

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Screening for transmission of hepatitis within a liver unit

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SUMMARY

Detailed screening of the patients and staff in a unit specializing in liver disease was carried out over a year to ascertain whether transmission of the serum hepatitis virus was occurring and whether the situation was comparable in any way to that found in a Renal Haemodialysis Unit. Of the 154 patients with liver disease tested on admission, 6% were found to have Australia antigen in the serum and throughout the year there were rarely less than two patients in the ward at any one time with positive serum. No instances of clinical hepatitis were detected in the other patients following their stay in the ward or in their attendant medical, nursing and lay staff. Six staff members were found to have Australia antigen in their serum. In four of these, all nurses, it was present in the first sample tested and so the infection may have been acquired earlier. Temporary elevations in both plasma bilirubin and serum aspartate aminotransferase levels were found in another five staff members whose serum was negative for Australia antigen and who clinically were well. In a further eight and apparently healthy staff members, an isolated but persistent elevation of the plasma bilirubin was noted. In both groups these changes could represent the spread of subclinical infectious hepatitis and it is recommended that in units dealing with 'liver patients' not only should considerable care be taken during diagnostic and therapeutic procedures but the medical and nursing staff should be screened at regular intervals.

INTRODUCTION

The outbreaks of hepatitis in renal haemodialysis units which have affected both staff and patients are currently the cause of considerable concern. In the majority of instances the cause has been serum hepatitis as shown by the finding of Australia antigen (Knight *et al.* 1970; Hawe, Goldsmith & Jones, 1971). Although the mode of entry into the unit has not always been clear, likely routes include blood transfusion and the admission of a patient who has had hepatitis previously and remained a carrier of virus. Spread within the unit subsequently is not surprising in view of the exposure to blood during haemodialysis procedures and shunt care. Furthermore, it has been shown that the serum hepatitis virus can be transmitted not only by the parenteral route but also orally (Giles, McCollum, Berndtson & Krugman, 1969) and possibly by droplet infection. The identification

of Australia antigen in faeces and in urine has also been reported (Apostolov *et al.* 1971). Much of the above, it seemed to us, would also apply to units in which patients with liver disease were cared for. A significant number of patients would be expected to have Australia antigen in their serum and the various procedures used in their treatment, for instance blood transfusion and the Sengstaken tube for bleeding varices, or exchange transfusion and extracorporeal pig liver perfusion in fulminant hepatic failure, all involve considerable exposure of both medical and nursing staff to the patients' blood. In order to ascertain the extent of the risk, for we could find no information in the literature, we decided for a 1-year period to screen all patients on admission as well as their attendant staff at regular intervals using both liver function tests and examination of serum for Australia antigen.

DETAILS OF SURVEY AND METHODS

The Liver Unit comprises a 22-bed ward of the old 'Nightingale' type partly partitioned to form cubicles containing three to six beds, one of them being immediately adjacent to the nursing station and used for patients requiring intensive care. The research laboratories and office accommodation are immediately adjacent to the patient area.

About two-thirds of the patients in the ward at any one time have liver disease, the remainder having general medical conditions. The aim was to test all patients both on admission and again on discharge, those staying in the ward for longer than a month also being tested at monthly intervals. All members of the staff including nurses, doctors, research fellows, students and the domestic and secretarial staff, all of whom had some contact with the patients, were screened at monthly intervals. Those visiting the ward only occasionally, such as radiographers and physiotherapists, were not included.

All blood samples were tested for Australia antigen and the associated antibody by the immunodiffusion technique. The sera from patients with fulminant hepatic failure were also examined for Australia antigen by complement fixation, immunoelectrophoresis and electron microscopy (Zuckerman, 1970). In the case of the staff, the liver function tests were also estimated in the same sample using the S.M.A. 12/60 Autoanalyser.

Precautions taken routinely when a patient with suspected or proven hepatitis was admitted to the ward and also for those patients found to be Australia antigen positive, included the use of disposable crockery and cutlery. When blood was taken or other clinical procedures carried out, gloves were always worn. The same precautions were taken for the patients with fulminant hepatic failure who in addition were barrier nursed in the intensive care area. Gamma globulin was not given to either staff or patients.

RESULTS

During the year, a total of 154 patients with liver disease and 108 patients with general medical conditions were tested on admission to the ward. These together represented nearly 80% of the total admissions over this period and accounted for

278 and 135 blood samples respectively. Particularly in the general medical patients further samples were often not obtained owing to a variety of reasons. A considerable number were emergency admissions and died shortly afterwards or were transferred to the care of other physicians and surgeons. There was also an appreciable early mortality in the liver disease group.

Neither Australia antigen nor the antibody was found in the serum of any of the general medical patients either in the initial or subsequent samples. An out-patient follow-up study carried out 3 months after their discharge from hospital also failed to reveal a single instance of clinical hepatitis that could have been contracted whilst in the ward.

Nine (6%) of the 154 patients with liver disease had Australia antigen in the serum on admission (Table 1). These included two patients with a primary hepatoma which had developed from an underlying cirrhosis. The antigen was found consistently in their serum throughout their stay in the ward. Of the remainder four patients had presumed serum hepatitis with a history of parenteral inoculation, three of them being drug addicts. The fourth patient with serum hepatitis and Australia antigen was a nurse who had been working in the Renal Haemodialysis Unit at Guy's Hospital during the outbreak of hepatitis there. The admission of these nine patients with positive sera had been fairly evenly spread through the year. The average duration of stay was 20 days, though one patient with positive serum was in the ward for over 2 months. Two other patients with presumed serum hepatitis, one of whom had been tattooed recently, were negative for Australia antigen, as were all those with the clinical diagnosis of infectious hepatitis.

Fulminant hepatic failure

The nine patients admitted during the year were treated by a variety of procedures involving direct contact of the medical and nursing staff with the patients' blood. Intravenous catheters were used for nutrition and measurement of central venous pressure, extracorporeal pig-liver perfusion and cross-circulation were

Table 1. *Results of testing for Australia antigen in the* 154 *patients with liver disease*

Diagnosis	Number of patients tested	Number of patients with positive sera
Serum hepatitis	6	4
Infectious hepatitis	9	0
Fulminant hepatic failure	9	2
Active chronic hepatitis	16	1
Primary biliary cirrhosis	15	0
Haemochromatosis	16	0
Alcoholic cirrhosis	11	0
Cryptogenic cirrhosis	12	0
Primary hepatoma with underlying cirrhosis	16	2
Hepatic secondary deposits	12	0
Other liver conditions	32	0
Total	154	9 (6%)

carried out once each, haemodialysis twice, exchange blood transfusion three times and in two cases a tracheostomy was performed. The cause of hepatic necrosis was paracetamol overdose in one patient and two cases had occurred shortly after a halothane anaesthesia. Of the remaining six patients one was known to associate with drug addicts. Australia antigen was found in her serum and in one other patient in whom no contact history could be elicited. In none of the cases could antibody to Australia antigen be detected.

Samples from the seven patients which were negative on immunodiffusion were also negative for Australia antigen when examined by complement fixation, immuno-electro-osmophoresis and electron microscopy. The sera were also examined for the Milan antigen by immunodiffusion using the specific anti-serum (Del Prete *et al.* 1970) but in no case was a positive reaction obtained.

Screening of staff

The 'turnover rate' amongst the nurses and medical students was high; few had more than two or three samples tested whereas most of the clinicians and research workers had at least six samples tested during the year (Table 2). No instance of a clinical illness compatible with acute hepatitis was encountered, but in five staff members serum was found to be positive for Australia antigen and in one other the antibody was detected. Four of these were nurses. One nurse was positive on the first occasion tested, subsequent samples being negative. The other three were Agency Nurses on temporary duty in the ward and each had only one sample examined. The fifth staff member with Australia antigen was a clinician, who was positive at the first testing and had two other positive samples over the subsequent 6 months. He was the only one with a history of infectious hepatitis in the past. This was 8 years previously and we could find no evidence of persisting hepatitis at the time he was on the ward. The one staff member with antibody to Australia antigen in the serum was also a clinician. He had positive serum on three occasions during the year, some of the intervening samples tested being negative. In none of these six people was the presence of Australia antigen or the antibody associated with any evidence of clinical illness and in each instance tests of liver function carried out at the same time gave normal results.

Table 2. *Results of serial screening in* 152 *members of staff*

	Clinicians	Medical students	Nurses	Lay staff
No. of subjects	33	32	66	21
No. of samples examined	225	79	140	86
No. of subjects with Australia antigen	1	0	4	0
No. of subjects with antibody	1	0	0	0
No. with raised plasma bilirubin and aspartate aminotransferase	1	3	0	0
No. with raised plasma bilirubin only	1	7	0	0

Abnormal liver function tests were, however, found in three students, one clinician and one secretary during the course of the year. In each instance the changes were slight with serum bilirubin levels of up to 1·4 mg./100 ml. and a serum aspartate aminotransferase (SGOT) of up to 125 mU/ml. The abnormalities were also temporary, the tests carried out on the sample taken a month later showing a return to normal values. A persistent elevation of the serum bilirubin was found in eight other staff members with levels of 1·8 to 3·2 mg./100 ml., the other liver function tests being within the normal range.

DISCUSSION

The frequency with which Australia antigen was found in the different varieties of liver disease admitted to the ward during the year is very similar to that reported in other series from this country, positive sera being found most frequently in cases of serum hepatitis and occasionally in active chronic hepatitis and primary hepatoma (Dudley, Fox & Sherlock, 1971). Both of the patients with primary hepatoma and an underlying cirrhosis could have contracted the infection abroad, for one was an immigrant from the West Indies whilst the other was known to have had serum hepatitis following a course of bismuth injections whilst in the Far East in 1943. We were surprised not to find more positive sera in the patients with fulminant hepatic failure due to a presumed viral hepatitis. Whether the severity of the hepatitis in such patients is a reflexion of a greater susceptibility of the patient or of greater virulence or dose of the infecting agent is not known, but Almeida & Waterson (1969) have suggested that Australia antigen may be present in the serum of these patients in the form of immune complexes. If so we should have been able to detect these on electron microscopy which was carried out in all of these patients.

Nevertheless, there was a substantial reservoir of patients with Australia antigen in the ward more or less consistently throughout the year. Though the extent to which the nursing and medical staff were directly exposed to the blood of patients is less in a Liver Unit than in a Renal Haemodialysis Unit there were certainly plenty of opportunities for transmission of infection to have occurred. In fact, there was no evidence of spread of Australia antigen from the liver patients to other patients in the ward. The four nurses found to have Australia antigen in their serum were all positive on their first testing and it is possible, therefore, that they had contracted the infection before coming to the ward. However, the one doctor with Australia antigen in the serum had been working on the ward for some time prior to the survey as had the other doctor whose serum was positive for the antibody. His first sample was negative so that it seems likely that both of these doctors had acquired the infection whilst working on the ward.

The temporary but simultaneous elevations of serum aspartate aminotransferase and serum bilirubin found in a number of the staff, but whose sera were negative for Australia antigen, are difficult to interpret. Such abnormalities in liver function could result from subclinical episodes of infectious hepatitis. More detailed investigation, such as by liver biopsy, which might have provided con-

clusive information, was not considered justifiable as the staff concerned were both free of symptoms and signs. The persistently raised serum bilirubin found in others of the staff could again be evidence of infection by hepatitis virus. An alternative explanation is that these subjects had Gilbert's Syndrome (mild congenital unconjugated hyperbilirubinaemia) but if so this would represent an unusually high incidence of that condition (Powell, Hemingway, Billing & Sherlock, 1967).

It must be stressed that although Australia antigen is identifiable with, or is at the very least a close marker of serum hepatitis, we have at the moment no specific test for infectious hepatitis and spread of the latter agent within the ward has not been excluded. The most recent studies on the Milan antigen, which was originally identified in three outbreaks of infectious hepatitis, have shown its presence in other liver diseases and its aetiological significance has been questioned by Taylor and her associates (1972). At the moment, therefore, it would seem only reasonable that in any ward where there is a significant number of 'liver patients' there should be a strict code of practice for dealing with blood samples and other aspects of their investigations and treatment. Patients should certainly be screened for Australia antigen on admission and the adoption of a policy for screening the staff at regular intervals has much to commend it.

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