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Aerobic bacteria occurring in the hind-gut of the cockroach, *Blatta orientalis*

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SUMMARY

Methods are described for the isolation and identification of aerobic bacteria occurring naturally in the hind-gut of the cockroach *Blatta orientalis* captured from a number of wild sources, to establish whether or not human pathogens occurred naturally within the gut. During the investigation an organism was frequently found which could not be classified in any described species, and for which we propose the name *Escherichia blattae*.

INTRODUCTION

Some 3500 species of cockroach have been described, the vast majority of which are of little or no significance to man. There are, however, perhaps seven species which are closely associated with man and commonly found breeding in buildings (Cornwell, 1968). Three of these, *Blatta orientalis*, the oriental cockroach or 'black beetle', *Blatella germanica* the German cockroach or 'steam fly', and to a lesser extent *Periplaneta americana*, the American cockroach, are spread throughout the British Isles.

These domestic cockroaches are all of tropical, probably African, origin (Rehn, 1945) and, in contrast to the three outdoor species of *Ectobius* found in Southern England, can survive only in a warm and a fairly humid environment such as is provided in kitchens, boiler-rooms and stores. Being essentially nocturnal in their feeding habits, cockroaches are often present in large numbers in buildings used during the day, and remain unnoticed, since they will secrete themselves in any available crack or crevice, behind furniture, underneath fixtures and around water pipes. It is only the occasional inadvertent wanderer, a dead body, a cast skin, or more dramatically a major structural repair to the building which reveals their presence.

The cockroach has been shown to feed readily on faeces, sputum, skin scrapings and other human waste, and on a wide variety of human foodstuffs (Roth & Willis, 1967). Cockroaches are ideally equipped to carry pathogenic organisms from an infected source to uncontaminated material, since they have hairs and bristles on legs and body, grasping claws and pads on their feet (although in our work this purely mechanical means of transmission was found to be of little significance), and their feeding habits involve considerable use of saliva, and indiscriminate defaecation. An impressive array of pathogenic organisms have been isolated from cockroaches living under natural conditions including *Escherichia*

coli, a number of *Salmonella* species, *Staphylococcus aureus* and the poliomyelitis virus (Roth & Willis, 1967). On at least three occasions we have found cockroaches in hospital kitchens, only a few yards away from refuse bins and surgical operating theatres, and we have isolated *E. coli* from these insects.

Under experimental conditions cockroaches have been shown to carry numerous pathogenic organisms externally, without multiplication of the organisms. But, more significantly, a variety of organisms, having been ingested, have been shown to multiply in the gut and appear in the faeces over a period of several days without loss of virulence. Examples of these are the cholera vibrio and plague bacillus (Barber, 1912), and *E. coli* (Steinhaus, 1941; Bitter & Williams, 1949). *Entamoeba histolytica* has also been isolated from cockroaches (Frye & Meleney, 1936) and the ova of a number of parasitic worms, for instance *Ancylostoma duodenale, Taenia saginata* and *Ascaris lumbricoides* (MacFie, 1922).

It is much more difficult to associate known outbreaks of disease with cockroach vectors since by their nature these diseases may be, and usually are, transmitted in a variety of ways. The evidence incriminating cockroaches may be purely circumstantial. In one striking incident an epidemic of food-poisoning in the children's nursery of a Brussels hospital subsided immediately an infestation of the German cockroach was controlled. *Salmonella typhimurium* was isolated from the insect (Graffar & Mertens, 1950). Other instances are reported by Antonelli (1943) and Mackerras & Mackerras (1948, 1949).

The natural infection of the cockroach with pathogenic organisms, and experimental evidence, coupled with the known domestication and unhygienic habits of the cockroach, make it, in the words of Roth & Willis, 'impossible to accept cockroaches as only minor annoyances of little medical importance'.

METHODS AND MATERIALS

Capturing the cockroaches

Forty cockroaches were investigated from six different sources as follows: twelve from the 7th floor kitchens of a large London hospital; five from the boiler-room of a smaller hospital; two from a paved yard and five from the staff canteen of the same hospital; six from a teaching college and ten from a long-established insectary culture. The wild cockroaches were caught on site between 11 p.m. and 2 a.m. using specially modified Petri dishes. The smaller half of a sterile dish was placed over the insect and the larger half, from which the rim over half the circumference had been removed, was slipped underneath, trapping the insect in the dish.

Dissection of gut and isolation of organisms

Dissection of the cockroach was carried out the next morning under sterile conditions. The legs, and wings if present, were removed, followed by the head. The sides of the abdomen were cut on either side of the anus, and the complete gut removed posteriorly. In four of the cultured insects, the fore-gut, mid-gut and hind-gut were treated separately, but in all the others only the hind-gut was investigated, the relevant portion being ruptured and emulsified in $\frac{1}{4}$ strength Ringer's solution. The emulsion was plated out on normal blood agar, 6 % blood

Table 1. *Gram positive bacteria isolated from cockroaches*

Organism	Number of isolates		Number of cockroaches in which organism occurred	Percentage of cockroaches with organism
Bacillus spp.	76		37	93
B. cereus gp.		1		
B. firmus		3		
B. licheniformis		6		
B. megaterium		4		
B. subtilis gp.		1		
B. coagulans		1		
B. pulvifaciens		5		
B. pantothenticus		2		
B. brevis		2		
B. circulans		5		
B. polymyxa		3		
Bacillus sp.		43		
Streptococcus spp.	53		28	70
S. bovis		2		
S. equinus		4		
S. durans		3		
S. faecalis		5		
S. faecium		1		
S. sanguis		5		
S. lactis		4		
S. cremoris		16		
Streptococcus sp.		13		
Staphylococcus spp.	11		9	23
Baird-Parker gp. II		3		
Baird-Parker gp. III		1		
Baird-Parker gp. IV		1		
Baird-Parker gp. V		4		
Baird-Parker gp. VI		2		
Micrococcus spp.	3		3	8
Baird-Parker gp. 6		1		
Baird-Parker gp. 7		2		
Aerococcus viridans	6		6	15
Unknown genera	8			

agar, MacConkey's agar and deoxycholate-citrate agar (DCA). After 24 hours' incubation the aerobic organisms were grouped, further tests carried out and identification achieved by use of Cowan and Steel's methods and tables (Cowan & Steel, 1965), except for the staphylococci where Baird-Parker's technique was used (Baird-Parker, 1963).

Biochemical tests were carried out using the following specific techniques: decarboxylase (Møller, 1955), citrate (modified Simmons, 1926), triple sugar iron agar (Report, 1958), indole (Kovacs, 1928), Hugh and Leifson's O-F medium (Hugh & Leifson, 1953), oxidase test (Kovacs, 1956), Voges-Proskauer (V-P) reaction (Barritt, 1936), malonate-phenylalanine medium (Shaw & Clarke, 1955) and gluconate (Shaw & Clarke, 1955).

Table 2. *Gram negative bacteria isolated from cockroaches*

Organism	Number of isolates		Number of cockroaches in which organism occurred	Percentage of cockroaches with organism
Enterobacteriaceae	54		30	75
Citrobacter freundii		11		
Enterobacter aerogenes		1		
E. cloacae		12		
Escherichia coli		2		
E. blattae		16		
Klebsiella edwardsii		2		
K. ozaenae		6		
Proteus vulgaris		1		
Serratia marcescens		3		
Acinetobacter anitratus	3		3	8
Pseudomonas aeruginosa	3		3	8
Unknown genera	2			

RESULTS

A total of 219 isolations was made, of which 157 were Gram-positive bacteria which were placed in 28 different species, and 62 were Gram-negative, placed in 11 species, including a new species which we have named *Escherichia blattae*. Results are shown in Tables 1 and 2.

DISCUSSION

It is often difficult to define the term 'normal flora', and to decide if it is made up of those organisms which occur most frequently and in the greatest numbers in a healthy animal, or whether it should include all organisms found in the healthy animal, ill-health being caused by abnormal flora. Again, the term 'healthy' is equally hard to describe when referring to an insect. If an insect is behaving normally, should we presume it is healthy?

None of the bacteria isolated from the cockroaches in this series of experiments appeared to have any deleterious effect on the animal. Indeed, in later experiments where human pathogenic Enterobacteriaceae were passaged through the cockroach, the insect survived admirably. The only fatalities occurred when *Serratia marcescens* was allowed to accumulate on MacConkey agar on which the cockroaches fed. From our work it appears that the insect will take up any organism in its environment. This was apparent when a series of *Klebsiella edwardsii* and *Proteus vulgaris* was isolated from insects from one locality but not from any other. *Acinetobacter anitratus* was isolated only from insects in an insectary culture. Species of *Hafnia*, *Enterobacter* and *Citrobacter* were isolated from the majority of insects investigated, and *Streptococcus cremoris* was very common.

In four cockroaches in which the bacteria of the fore-gut, mid-gut and hind-gut were investigated the variety of species increased from fore-gut to hind-gut, there being, on average, two species in the fore-gut, three in the mid-gut and five in the

Table 3. *Correlation between* Escherichia blattae *and similar organisms*

	Esch. spp.	Esch. blattae 1	Esch. blattae 2	Hafnia alvei	Enterobacter spp.	Serratia marcescens
Citrate	− (0·8 % +)	−	+	+	+	+
Motility	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Indole	+ (1·0 % −)	−	−	−	−	−
H_2S	−	−	−	−	−	−
Gluconate	−*	+	+	+	+	+
PPA	−	−	−	−	−	−
Gelatin	−	−	−	−	+	+
Lactose	+ (9·5 % −)	−	−	−	+	d
ONPG	+	−	−	+	+	+
Sucrose	d	−	−	d	+	+
Mannitol	+ (1·0 % −)	−	−	+	+	+
VP	−	−	−	d	+	+
Arginine	d	+	+	−	d	−
Malonate	−	−	+	+	d	d
KCN	−	−	−	+	+	+

Percentage variations in parentheses.
* Except *Esch. adecarboxylata* and *Esch. blattae*.

Table 4. *Biochemical reactions of three species of* Escherichia

	Esch. coli*	Esch. blattae	Esch. adecarboxylata†
Motility	+	+	+
KCN	−	−	+
Glucose (gas)	+	+	+
Lactose (acid)	+	−	+
Mannitol (acid)	+	−	+
Sucrose (acid)	d	−	+
Dulcitol (acid)	d	−	+
Inositol (acid)	−	−	.
Adonitol (acid)	−	−	.
Arabinose (acid)	+	+	.
Malonate	−	d	+
Indole	+	−	+
MR	+	+	+
VP	−	−	−
Citrate (Simmons)	−	d	−
Urea	−	−	+
H_2S (TSI)	−	−	−
Gelatin	−	−	+
Phenylalanine	−	−	−
Lysine decarboxylase	d	+	−
Ornithine decarboxylase	d	+	−
Arginine dihydrolase	d	+	−
Gluconate	−	+	+

* Cowan & Steel (1965).   † Leclerc (1962).

hind-gut. As the pH value is known to increase from fore-gut to hind-gut increased acidity may have a bactericidal effect.

Escherichia blattae

Sixteen biochemically similar organisms of the 54 Enterobacteriaceae isolated could not be placed in any accepted group. The organism which we have named *Escherichia blattae* appeared as two biotypes, one of which was citrate and malonate positive, the other negative. In every other respect the two varieties were identical. The organism closely resembled *E. coli*, except for a positive gluconate reaction. However, a gluconate positive *Escherichia*, namely *E. adecarboxylata*, has been described (Leclerc, 1962). Apart from *E. coli*, the organism resembled most closely the following motile gluconate positive species: *Hafnia alvei*, *Enterobacter* spp. and *Serratia marcescens*. Correlation with all the above organisms was strengthened by negative PPA and H$_2$S reactions.

As can be seen from selected tests shown in Table 3, correlation between *E. blattae* and *Escherichia* spp. is greater than that between *E. blattae* and the other organisms shown, bearing in mind especially the modern view that lactose-negative strains of *Escherichia* are acceptable. Although there was a close correlation between *E. blattae* and *Hafnia alvei*, numerical identification confirmed a closer relationship to *Escherichia*. The organism was tested against all known *E. coli* sera and no cross-reactions were detected. Percentage variations of reactions of *Escherichia* spp. shown in Table 3 are quoted from Edwards & Ewing (1962).

Table 4 shows biochemical reactions of *E. blattae* compared with *E. coli* and *E. adecarboxylata*.

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Laboratory transmission of Enterobacteriaceae by the oriental cockroach, *Blatta orientalis*

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SUMMARY

Methods of maintaining and feeding and of infecting cockroaches with pathogenic organisms were investigated.

Cockroaches fed on known concentrations of *Escherichia coli* O119, *Esch. coli* O1, Alkalescens Dispar O group 2 and *Shigella dysenteriae* 1 (Shiga's Bacillus) were maintained in Petri dishes. The effect of various diets on the survival of these organisms within the hind-gut and faeces was observed. With a 'normal' diet of gruel *Esch. coli* O119 was isolated for up to 20 days, *Esch. coli* O1 for 17 days and ADO 2 for 15 days. *Sh. dysenteriae* 1 was isolated only sporadically to the third day.

INTRODUCTION

The domesticated cockroaches, *Blatta orientalis, Periplaneta americana* and *Blattella germanica* have been shown to feed readily on faeces, sputum, skin scrapings and other human waste, and a wide variety of human food-stuffs (Roth & Willis, 1967). Burgess, McDermott & Whiting (1973) investigated the normal aerobic bacterial flora of the hind-gut of *Blatta orientalis* and refer to a number of instances where pathogenic bacteria have been isolated from cockroaches, both under natural conditions and in laboratory passage experiments.

METHODS AND MATERIALS

Maintenance of cockroach colonies

Later stage nymphs and adult cockroaches were housed individually in sterile disposable Petri dishes. Three series of cockroaches, 59 cockroaches in each series, were fed as follows.

Series A were given only water each day; these are termed 'starved'.

Series B were provided each with half of a 9 cm. filter paper for shelter, and were fed on a 'normal' diet of a sloppy chicken food gruel emulsified in water.

Series C were kept on sterile MacConkey plates agar downwards.

Plates were changed and individuals fed or watered every 24 hr.

Infection of cockroaches with organisms

A suspension containing a known number of organisms determined by the technique of Miles & Misra (1938) was used. After 72 hr. starvation, each cockroach was placed overnight in an incubator at 35° C. Next morning, one drop of a known

concentration from a 50-drop Pasteur pipette was placed with the cockroach in a sterile Petri dish. The majority of cockroaches drank readily. Only those which took the full quantity were used subsequently. Each series of examinations included ten uninfected cockroaches as controls.

After ingestion one cockroach from each of the three feeding groups was killed daily and the hind-gut examined for the presence of the specific organisms. The cockroach was dissected under sterile conditions, the hind-gut being removed and emulsified in $\frac{1}{4}$ strength Ringer's solution. The emulsion was plated out on MacConkey's medium, incubated at 37° C. for 24 hr., and any growth observed.

In addition, the MacConkey plate on which the cockroach had lived and defaecated for the 24 hr. before dissection was incubated at 37° C. overnight, and any growth recorded.

During preliminary work it was suspected that the organism, or an associated toxin, was killing the infected cockroach. To check this, groups of ten cockroaches were fed as follows by the method described above.

(a) An overnight growth of Esch. coli O119 in nutrient broth (i.e. organisms with metabolic products and broth).

(b) Broth from an overnight growth, centrifuged, Seitz filtered, and checked for sterility (i.e. broth with metabolic products but without organisms).

(c) Organisms from the overnight growth washed four times and resuspended in fresh nutrient broth (i.e. organisms with broth but with no metabolic products).

(d) Fresh sterile broth.

A group of ten cockroaches served as a control. Only two died, one in group c and one in the control group. Since a damaging immobilization and forced feeding technique had been used in preliminary work, it was concluded that the method of infection, and not the organism or an associated toxin, had killed the cockroaches.

Number of organisms in infecting dose

To determine the optimum concentration of organisms, cockroaches were fed 0·2 ml. of Esch. coli O119 organisms at concentrations ranging from $5·5 \times 10^3$ to $5·5 \times 10^8$ per ml. The organism was recovered from cockroaches fed on all concentrations 3 hr. after ingestion, but thereafter, for up to 10 days, only from cockroaches fed on the highest concentration. It was decided to infect the cockroaches with a high concentration of each organism. The concentrations used were as follows: Esch. coli O119 2×10^8/ml., Esch. coli O1 $2·8 \times 10^8$/ml. and ADO 2 $3·1 \times 10^8$/ml. Each cockroach imbibed 0·02 ml. of the suspension (1 drop from a 50-dropper pipette).

MacConkey agar was used for isolation of Esch. coli and Alkalescens Dispar, deoxycholate citrate agar for the isolation of Sh. dysenteriae. Representative samples of colonies were selected, and shown to be pure by subculturing three times on blood plates. Identification was confirmed by Preston and Morrell's modification of Gram's stain (Preston & Morrell, 1962), followed by 'short sets' of biochemical tests consisting of Simmons' citrate agar (Simmons, 1926), TSI slopes (Report, 1958), peptone water (indole test) (Kovacs, 1928), nutrient agar and

Table 1. *Isolation of three organisms from the hind gut of cockroaches after experimental infection*

	Escherichia coli O119	*Escherichia coli* O1	Alkalescens–Dispar O group 2
Series A Starved	13* (15)	8 (16)	8 (15)
Series B Gruel-fed	16 (20)	12 (17)	15 (15)
Series C MacConkey agar	13 (20)	10 (17)	13 (18)

* The figures show the number of days for which the organism was grown at each successive daily examination. The figures in parentheses show the last day after infection on which a positive result was obtained.

nutrient broth (for serological testing), gluconate broth (Shaw & Clarke, 1955) and urea slopes (Oxoid CM 71) (Christensen, 1946). After biochemical identification specific antisera (from David Bruce Laboratory, Everleigh) were used to confirm identification. Results will be discussed individually.

RESULTS

Esch. coli O119

The organism was isolated from the hind-gut of the cockroaches 24 hr. after infection and continued to be isolated for 15 days in the starved group (Series A) and for 20 days in the gruel fed group (Series B) and the MacConkey fed group (Series C).

Esch. coli O1

Cockroaches easily became infected with this organism and it was isolated from all three feeding series during the first day after infection. In Series A given only water, the organism disappeared on the 8th day except for one colony which was isolated on the 16th day. In Series B fed on gruel, isolations were made regularly to the 12th day, remaining fairly constant to the 17th day. In the MacConkey Series C isolations were regular to the 10th day and somewhat sporadic thereafter to the 17th day.

ADO 2

This organism also was isolated during the first 24 hr. after infection in all series. In the starved Series A it continued to be isolated up to the 8th day; beyond this a very high death rate, some 50 % higher than in either of the other starved series, renders results unreliable. In the gruel fed Series B, the organism continued to appear up to the 15th day, and in the MacConkey Series C isolations were regular to the 13th day.

These results are shown in Table 1.

Sh. dysenteriae

Groups of ten cockroaches were fed with five different concentrations of *Sh. dysenteriae* in suspension, ranging from 8×10^3 to 8×10^7 organisms/ml. Two cockroaches from each group were dissected on days 1, 3, 12, 22 and 30 after infection. The ten insects dissected and plated on day 1 were all negative; two isolations were made on day 3, one from a concentration of 80 million organisms per ml. and the other at 8000 per ml. After the third day, no isolations were made. Further work is clearly necessary on the ingestion of this organism.

DISCUSSION

Mechanism of transmission of organisms

The infecting organisms were isolated also from cockroach faecal pellets and smears deposited on the MacConkey plates some 24 hr. *before* dissection with an incidence similar to that occurring in later hind-gut cultures. Clearly it is more practical to isolate organisms from faeces provided by the cockroach than to dissect out the hind-gut.

Artificially maintained cockroaches show a number of features which would be encountered in free living cockroaches.

Cultured cockroaches, given only water, voided almost fluid faeces but retained considerable solid matter in the hind-gut which was apparent on dissection. Those fed on gruel produced numbers of faecal pellets rather than smears. When the faecal pellet was spread a confluent growth occurred. Contamination of the MacConkey plate was directly proportional to the length of time the surface had been exposed to the insect. Little contamination occurred when the cockroach was kept on the plate for only 6 hr. Ten minutes exposure produced growth rarely, whereas 24 hr. produced a good growth. Clearly, the deposition of fluid faeces on a moist medium affords the best conditions for the survival and growth of organisms. It is significant that on transferring the cockroach from one plate to another, little or no growth of organisms occurred unless faeces had been present, in which case the organisms grew in abundance. The normal flora of the cockroach was never suppressed by growth of the inoculated organism. Cockroaches fed on MacConkey medium did not produce faecal pellets and the hind-gut on dissection contained little faecal matter. The internal tissues of cockroaches in this group were stained with neutral red. Growth of organisms on the plate after incubation occurred mainly from the faecal streaks. The heaviest mechanical spreading occurred on the circumference of the plate where the insect spent most of its time, and thus dispersed its excreta.

Death rates over a period of 20 days after the initial starvation depended significantly upon diet, since no cockroach died which was fed on a 'normal' diet of gruel. There was a high death rate in those given water only (39%) and a moderate death rate in those fed on MacConkey medium (27%). Although the initial treatment of starvation and incubation resulted in a number of deaths (23%) it did not appear to affect subsequent survival.

A problem which occurred in the group fed on MacConkey medium was caused

by the organism *Serratia marcescens* which, when present in the faeces of the cockroach, grew on the plate at room temperature. The growth was re-ingested by the cockroach, defaecated onto a new plate, re-ingested and so on until eventually the concentration of *S. marcescens* appeared to kill the cockroach. This organism is present in the normal gut flora of some 6% of the insects used (Burgess, McDermott & Whiting, 1973), and under certain circumstances might present a means of biological control. In low concentrations the organism will probably fail to survive in the gut, but at higher concentrations the defence mechanism of the cockroach may be overwhelmed. This would also appear to happen in infection of the cockroach with the strains of *Esch. coli* used. Though low concentrations were quickly evacuated during the first few hours, high concentrations appeared to become established. In our investigation into the normal flora of *Blatta orientalis* we isolated *Esch. coli*, from which it must be concluded that the organism must have existed in a high concentration in the material on which the cockroach had fed.

It is worth comparing the habits of the three domestic cockroach species when considering them as possible disseminators of pathogenic organisms. *Blatta orientalis* typically shows a dislike of climbing to table tops and other raised surfaces, but will often be found on the upper floors of high buildings.

Its density is usually far lower than that of its more mobile competitor *Blattella germanica* which seems to thrive in new buildings, as well as the older buildings, boiler rooms and cellars preferred by *Blatta orientalis*. We have observed an interesting take-over of a canteen by *Blattella germanica*, which has almost completely ousted *B. orientalis* in a period of just over two years.

Periplaneta americana appears to be of little importance so far in this country. It has, however, been found by us in London sewers, contrary to statements in, for example, Cornwell (1968, p. 304).

Taking all facts into consideration it would seem that, of all cockroaches, *Blattella germanica* is likely to prove the most important potential disease vector, but the capabilities of the less mobile and less numerous *Blatta orientalis* should not be underestimated.

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The Hampshire epidemic of foot-and-mouth disease, 1967

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SUMMARY

An analysis was made of the spread of foot-and-mouth disease during the epidemic in Hampshire in January and February 1967. To explain the pattern of spread, it had to be postulated that virus was present seven days before the first outbreak was reported. It is suggested that the disease occurred initially in pigs fed on infected meat and that the virus was subsequently disseminated from the local abattoir, where the pigs were killed, to four farms by movement of animals, slaughterhouse waste, people or vehicles, and to fifteen by the airborne route. Subsequent spread from these farms was by movement in two instances and by the airborne route in five. The source and route of infection of the last farm in the outbreak were not determined.

The risk of spread through movement was associated more with carriage of infected slaughterhouse waste, movement of animals, people or vehicles carrying animals than through collection of milk, artificial insemination or movement of other types of vehicles. Outbreaks of disease among pigs gave rise to more secondary spread than outbreaks in cattle. Secondary outbreaks attributed to airborne spread occurred only in ruminants. Most airborne spread was into areas of high livestock density and cattle in the larger herds became infected. Airborne spread could be correlated with wind direction and speed but not with rain. The reduction in the number of outbreaks at the end of the epidemic could be attributed to the elimination of the largest sources of virus, the control of movements and the fact that in all instances except two the wind was blowing virus over towns and out to sea, to areas of low stock density and to areas where animals had been killed.

INTRODUCTION

As a result of the epidemic in Great Britain in 1967–1968 a number of studies of the epidemiology of foot-and-mouth disease in that epidemic and in others has appeared (Henderson, 1969; Smith & Hugh-Jones, 1969; Wright, 1969; Hugh-Jones & Wright, 1970).

A series of foot-and-mouth disease outbreaks due to strain O_1 occurred in Hampshire between 6 January and 3 February 1967. The disease was confirmed on 29 farms and 2774 cattle, 414 sheep, 4708 pigs and six goats were slaughtered. Of these, 170 cattle, 285 pigs and four sheep had developed foot-and-mouth disease (Report on the Animal Health Services in Great Britain, 1967). The same report described the spread of disease (see later). Smith & Hugh-Jones (1969)

examined the epidemic in detail and reported on the weather elements involved. In the light of recent findings on the factors involved in airborne spread of foot-and-mouth disease (Sellers & Parker, 1969; Donaldson, Herniman, Parker & Sellers, 1970; Sellers, Donaldson & Herniman, 1970; Sellers, 1971; Sellers, Herniman & Donaldson, 1971; Barlow, 1972; Donaldson, 1972; Sellers & Herniman, 1972), we decided to re-examine the course of the epidemic.

In the paper we quote first the description given in the Report on Animal Health Services; second, the results of Smith & Hugh-Jones (1969); and, third, our version of the course of spread. The remainder of the paper is devoted to the presentation of the data available, to the arguments for our interpretation and to conclusions that can be drawn on the nature of the spread.

The Hampshire epidemic – the spread of the disease
(Report on Animal Health Services in Great Britain, 1967)

'On 6 January foot-and-mouth disease was confirmed in cattle at Southwick, Hampshire (1).* Lesions were recent in the two affected animals, and at the time of confirmation there was no indication of the source of infection.

On 7 January two further outbreaks (2, 3) were confirmed within 2 miles of the original case. There was no apparent connexion between the three cases, all of which were of recent occurrence, and consequently it seemed probable that an undisclosed source of disease existed in the district.

On 8 January recent disease was confirmed in a large number of pigs in a swill-fed herd (4) some 3 miles distant from the first outbreak. This proved to be the primary case. Infection was considered to have been introduced in waste food which could have contained imported meat scraps.

A further outbreak was confirmed on 9 January in pigs at a local abattoir (9). It was found that pigs from the swill-fed herd (4) had been moved to this abattoir on 3 January. They were slaughtered on the same day but the affected pigs at the abattoir had occupied the same pens between 4 and 9 January, and had presumably contracted disease from the infection left in the pens by the swill-fed pigs. These were undoubtedly shedding virus although showing no clinical signs of foot-and-mouth disease.

Four farms (5, 11, 14, 16) became infected through the movement either of animals, persons or vehicles from the abattoir on the days following the slaughter on 3 January of the pigs from the primary case. Disease also occurred on one farm (13) which had received offal and meat trimmings from the abattoir for boiling before feeding to pigs.

In addition there were 18 cases of disease which occurred in the vicinity of other outbreaks, or were on the route taken by the vehicles which conveyed the pigs from the swill-fed herd to the abattoir on 3 January. Foot-and-mouth disease was also confirmed in the swill feeder's other herd of pigs (6).

Infected area. The usual 10-mile radius infected area restrictions were imposed around the initial outbreak on 6 January.'

* The numbers in brackets refer to the farms listed in Table 1.

The weather factor in foot-and-mouth disease epidemics
(Smith & Hugh-Jones, 1969)

'We have examined four epidemics of foot-and-mouth disease in detail, those in Hampshire (1967), Northumberland (1966), Cheshire (1952) and Oswestry (1961). As will be seen, the feature common to the initial stages of each of them was the apparent relative inability of the disease to spread upwind. In the early stages of the epidemic in Hampshire in 1967 several outbreaks were traced from the movement of vehicles to and from the abattoir at Fareham. The first outbreak (1) was confirmed on 6 January and the two cows affected were slaughtered the same day, but by the morning of January 7 there were probably seven sources of virus (2–8) in existence. The largest of these was one of 141 infected pigs (4) in which the disease probably started on 3 or 4 January (confirmed on 8 January). Between 08.00 hr. on 3 January and 16.00 hr. on 6 January, the surface wind lay between 280° and 360°, that is, in the quarter between west and north. There was no rain during this period and there was no subsequent spread of the disease to the south-east. But on the evening of 6 January it began to snow, later turning to rain, during which the wind backed from 210° to 060° at dawn. These directions determine the rain–wind sector of the figure centred on the outbreak among 141 pigs (4). All the subsequent outbreaks occurring within the next 15 days lay within this sector.'

Our version of the origin and spread of disease

We suggest that infection was present in the area before 3 January and probably on 29 December. The primary outbreak was one of the following: the swill-fed herd (4) (most likely), the local abattoir (9), or the farm which had received offal and meat trimmings (13). Movement to and from the abattoir accounted for five or at the most eight outbreaks. The abattoir was probably a source of airborne virus from 31 December until 9 January. From 31 December until 6 January the wind was from the west and north-west and there was spread downwind to nine (1, 2, 3, 5, 7, 8, 10, 11 and 12) or at the least six outbreaks. On 6 and 7 January the wind from the south-west gave rise to possibly one outbreak (25). From 7 January to 9 January the wind was from between the north and east and there were five (19, 21, 22, 23, 26) or possibly six outbreaks downwind. The swill-fed herd (4) gave rise to one further outbreak (6) due to movement of people and swill. The farm which had received offal (13) was a source of infection for one farm due to movement of one person (18) and for three farms by the airborne route (17, 25, 27). The remaining outbreaks were due to airborne spread from other infected farms (10 → 20, 16 → 24, 24 → 28) or due to unknown causes (29).

Sources of information

1. The files on each infected premises and the summaries, maps and plans prepared by the Animal Health Division of the Ministry of Agriculture, Fisheries and Food.

2. The weather records of surface wind direction and strength, precipitation and relative humidity from the RAF Station at Thorney Island, Hampshire, from 26

Table 1. *Farms, dates and animals affected during the Hampshire epidemic*

Out-break no.	Date of reporting	Stock				Number affected	Suggested earliest date of disease
		C*	S*	P*	G*		
1	5. i. 67	86	—	21	—	2 c*	5. i. 67
2	6. i. 67	123	—	—	—	5 c	5. i. 67
3	7. i. 67	93	39	—	—	17 c 4 s	6. i. 67
4	8. i. 67	—	—	508	—	141 p	29. xii. 66†
5	8. i. 67	139	—	—	—	2 c	8. i. 67
6	9. i. 67	61	10	106	—	4 p	6. i. 67†
7	9. i. 67	45	—	—	1	4 c	9. i. 67
8	9. i. 67	130	—	—	—	12 c	8. i. 67
9	9. i. 67	60	114	226	—	10 p	31. xii. 66†
10	9. i. 67	106	—	—	—	11 c	9. i. 67
11	9. i. 67	66	—	—	—	4 c	8. i. 67
12	10. i. 67	84	—	100	—	22 c	9. i. 67
13	10. i. 67	125	—	473	—	126 p	6. i. 67†
14	11. i. 67	—	—	617	—	1 p	11. i. 67
15	11. i. 67	69	1	621	—	10 c	10. i. 67
16	11. i. 67	144	39	29	—	5 c	10. i. 67
17	11. i. 67	76	—	99	—	1 c	10. i. 67
18	12. i. 67	—	—	14	—	3 p	10. i. 67†
19	12. i. 67	97	—	—	—	4 c	11. i. 67
20	14. i. 67	128	—	—	—	1 c	13. i. 67
21	14. i. 67	14	—	9	—	2 c	13. i. 67
22	15. i. 67	49	—	—	—	2 c	14. i. 67
23	15. i. 67	49	—	—	—	4 c	14. i. 67
24	16. i. 67	134	—	149	—	8 c	15. i. 67
25	17. i. 67	69	—	—	—	21 c	16. i. 67
26	17. i. 67	94	—	—	—	1 c	16. i. 67
27	20. i. 67	236	—	547	—	10 c	19. i. 67
28	26. i. 67	98	—	56	—	8 c	24. i. 67
29	3. ii. 67	16	—	—	—	10 c	27. i. 67†

* C, c = cattle; S, s = sheep; P, p = pigs; G, g = goats.
† Lesions over 48 hr. old reported.
Note. In addition, 363 cattle, 211 sheep, 1097 pigs and 5 goats were slaughtered as 'direct contacts'.

December 1966, to 4 February 1967. Records of wind direction and strength from Calshot Meteorological Station, South Farnborough Meteorological Station, Hurn Airport and Southampton Weather Centre for 6 and 7 January 1967.

3. Visits to the areas and interviews with Divisional Veterinary Officers, Veterinary Officers and others concerned with the outbreak.

Farms, number of stock, number of outbreaks and date of reporting

Statistical information about the outbreaks is shown in Table 1. The farms are given in chronological order of reporting disease.

Topography of the outbreak

A map of the area and the site of the outbreaks are shown in Fig. 1.

Excluding the outbreak at farm 14, the area covered was 26 km. east–west by 16 km. north–south. Most of the outbreaks occurred in the valley of the Wallington

Fig. 1. Map of the area involved in the epidemic showing rivers, 200 ft. contours, towns and farms involved. Cross-hatching indicates built-up areas. S = Southampton, F = Fareham, G = Gosport, P = Portsmouth, W = Waterlooville, H = Havant. 1–29: Farms with animals infected. No. 14 is not shown but lies north-west of No. 16.

river, which on the west joins with the Meon Valley at Funtley. To the south lie the Portsdown and Fareham ridges and to the north the Hampshire Downs. Southampton lies to the west, Fareham and Portsmouth to the south and Havant to the east of the area.

Movement of animals, vehicles, people or materials between premises

The movements are shown in Table 2. The abattoir (9) was, as to be expected, the centre with which most movement was concerned. In two instances, potentially infected slaughterhouse waste and animals were taken to farm 13 and farm 16 respectively; in the other cases, people visited the abattoir or the animals were brought for slaughter. Thus, if virus were carried back to the farm, it would be on the visitors or in their vehicles.

Farms 2, 3, 5 and 10 were visited by inseminators. The only possible transfer of virus to initiate disease would have been from farm 2 to farm 10 on 4 January, before the cattle at farm 2 had developed lesions on 5 January. This would give an incubation period of 5 days. For any other implication of inseminators the incubation period would be too short (between farms 2 and 3) or no virus could have been present in the animal (between farms 2 and 5).

Table 2. *Movement of animals, vehicles, people or materials between infected farms*

Farm no.	Movement
9	From Farm 4: 44 pigs, 29 December; 65 pigs, 3 January
	From Farm 2: Visit, 2 January
	From Farm 5: 1 calf, 2 January; 2 calves, 6 January
	From Farm 11: 6 cows, 5 January
	From Farm 13: 2 pigs, 30 December. Slaughterhouse waste collection daily till 5 January
	From Farm 14: 10 pigs, 29 December. Visit, 2 January, 2 pigs, 4 January
	From Farm 15: 7 pigs, 2 January
	From Farm 16: 8 cattle, 2 January. Visit, 4 January, 1 bullock, 5 January (subsequently taken back to Farm 16 with seven other cattle)
2	A.I. visits on 1, 2, 3, 4 and 5 January
3	A.I. visits on 1 and 5 January
4	Daily visits to Farm 6
5	A.I. visit on 1 January
10	A.I. visits on 1, 2 and 4 January
11	Relief milker from Farm 5, 2 January
14	Father of owner of Farm 13 visited, 7 January
18	Owner worked at Farm 13 till 7 January
29	Cattle broke out on 28/29 January

Milk collections: (A) Farms 5, 11, 7
               (B) Farms 8, 10, farm, 13
               (C) Farms 15, three farms, 19
               (D) Farms 17, 26, 22
               (E) Farms 24, farm, 28

Although a number of farms were on the same milk collection route, FMD was found on all these farms within one day (A, in Table 2), the same day (B) and one day (3), i.e. too short an incubation period. A possible spread by infected milk within a reasonable incubation period might have occurred from farm 17 to farms 22 and 26 (D) and from farm 24 to farm 28 (E).

Meteorological conditions

The surface wind bearing and strength at Thorney Island from 26 December 1966, to 4 February 1967, are shown in Table 3. From 28 December till 6 January the surface wind came from westerly sectors between south and north (180°–360°) with speeds of up to 26 knots. During the night of 6/7 January the wind veered to 70° and from then until 9 January came from the north-easterly sector. Subsequent sectors were north-westerly, 350°–170°, southerly and westerly.

On the night of 6/7 January the records of surface winds at Calshot were similar to those at Thorney Island, whereas at Southampton and South Farnborough there was a period of calm between winds from the south-east and winds from the north-east.

Rain and wind speed from 29 December until 16 January (except 13 and 14 January) are shown in Table 8.

Table 3. *Wind bearing and strength at Thorney Island before and during the epidemic*

Period		Wind bearing	Wind speed (knots)
26. xii. 66	01.00–06.00	70°–80°	5–7
26–27. xii. 66	07.00–13.00	150°–200°	7–21
27–28. xii. 66	14.00–01.00	280°–300°	5–9
28. xii. 66	02.00–06.00	10°–80°	Nil–1
	07.00–17.00	180°–250°	4–17
28–30. xii. 66	18.00–10.00	220°–280°	1–25
30–31. xii. 66	11.00–01.00	180°–240°	5–18
31. xii. 66	02.00–08.00	230°–250°	20–26
31. xii. 66– 1. i. 67	11.00–22.00	250°–290°	6–26
1–2. i. 67	23.00–24.00	290°–330°	2–12
3–6. i. 67	01.00–14.00	290°–360°	0–15
6–7. i. 67	16.00–05.00	190°–320°	1–14
7. i. 67	05.00–06.00	320°–70°	7–14
7–9. i. 67	07.00–04.00	10°–120°	6–15
9–15. i. 67	05.00–12.00	250°–360°	Nil–11
15–16. i. 67	13.00–24.00	350°–170°	1–11
17–20. i. 67	01.00–24.00	150°–220°	7–22
21–24. i. 67	01.00–10.00	150°–300°	Nil–27
24. i. 67	11.00–22.00	230°–360°	Nil–10
25–26. i. 67	02.00–24.00	120°–290°	Nil–24
27. i. 67	01.00–10.00	230°–270°	7–12
27–28. i. 67	11.00–24.00	190°–240°	8–16
29–31. i. 67	01.00–07.00	110°–200°	4–19
31. i. 67– 4. ii. 67	08.00–03.00	230°–290°	4–23
4. ii. 67	04.00–23.00	290°–360°	2–9

Virus output

Estimates of the daily amount of airborne virus put out from 5 January to 3 February are shown in Fig. 2. One infected pig was assumed to have produced at least 30 times as much airborne virus as one cattle or one sheep (Sellers & Parker, 1969), although in practice this may have been higher. Cattle or sheep were estimated to produce 10^2 ID50 per minute and this represents one unit in Fig. 2.

Considerations

Incubation period

(a) *Direct and indirect contact.* In experiments at Pirbright with the O_1 strain of FMD virus, the range of onset of disease in animals housed in the same box or isolation unit was 2–4 days for pigs and 3–6 days for cattle.

(b) *Feeding of pigs with infected material.* One of 30 pigs fed on liver and bone from a steer infected with an O strain of FMD virus showed lesions on the fourth day (Henderson & Brooksby, 1948). Since a small dose was given in this experiment, the incubation period for pigs infected by feeding on swill was taken to be 3–4 days.

(c) *Artificial insemination.* One heifer artificially inseminated with $2 \cdot 5 \times 10^6$ mouse ID50 of virus of an O strain had clinical signs of FMD on the second day (Cottral, Gailiunas & Cox, 1968).
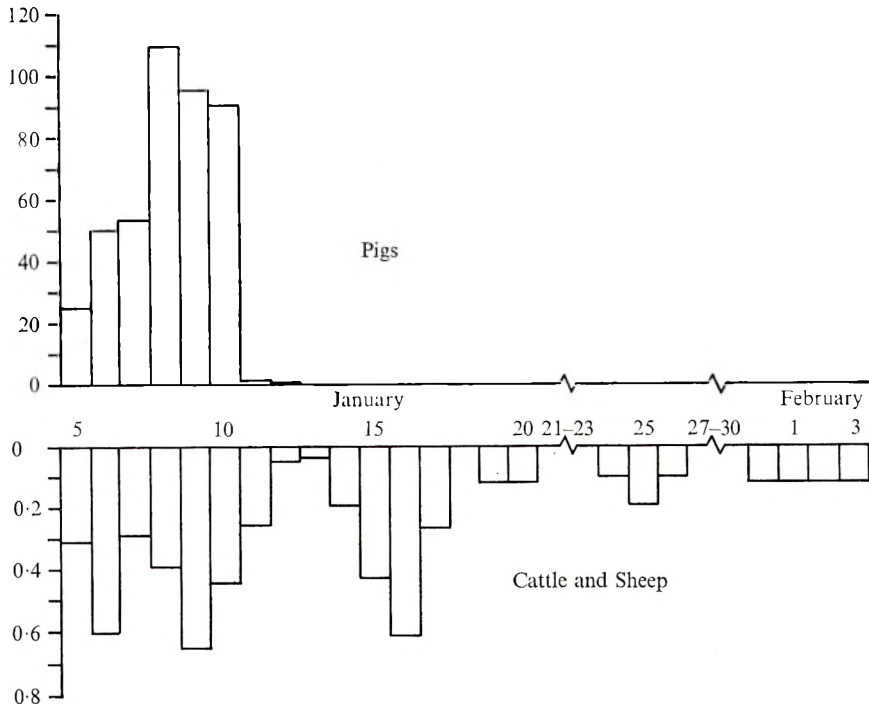
Fig. 2. Estimated relative amounts of airborne FMD virus produced by pigs and by cattle and sheep from 5 January till 3 February 1967. The amounts were calculated from the evidence found at slaughter, so do not include any virus that might have been produced before 5 January. It should be noted that the scale for cattle and sheep is a hundred-fold less than that for pigs. Cattle and sheep were estimated to produce 10^2 ID_{50} per minute.

(d) *Intramammary*. Cows given O_1 virus by the intramammary route developed generalized disease on the third day (Burrows *et al.* 1971).

(e) *Transfer by man*. Where cattle were infected by being breathed on, sneezed, coughed or talked at by people who had virus in their noses, the incubation period was 13–14 days (Sellers, Herniman & Mann, 1971). The estimated dose the animal received was 100 ID 50.

(f) *Airborne between farms*. The shortest incubation period for cattle in the same unit was 3 days; we have therefore assumed that, where cattle subsequently had disease, the incubation period between farms could vary from 4 to 10 days with a possibility of 14 days at the end of the epidemic when the amount of airborne virus emitted was likely to be low.

Route of infection

Cattle are more readily infected through inhalation of material containing virus than by ingestion (see Sellers, 1971) and this is probably also true of sheep. Pigs are difficult to infect intranasally (Graves & Cunliffe, 1960); on the other hand, when they are feeding on infected swill they are likely to take the virus in both by inhalation and by ingestion. Where different species are equally at risk, cattle are likely to receive a larger dose than sheep or pigs and show the disease first (Sellers

& Parker, 1969). Thus, pigs are likely to be infected through feeding on infected swill, through contact with infected pigs, through movement in contaminated vehicles or through contact with people contaminated with virus. Cattle and sheep are likely to be infected by inhalation in the open air or in buildings, in contaminated vehicles or by inquisitive sniffing of people contaminated with virus.

Source of virus

Pigs produce more airborne virus than cattle or sheep (Sellers & Parker, 1969; Donaldson *et al.* 1970). Cattle produce high titres of virus in milk and in faeces (Hedger & Dawson, 1970; Parker, 1971).

THE EPIDEMIC

Table 4 gives the bearings and distance of various farms from the abattoir (9), farm 13 and other farms (1, 10, 16 and 24). The date of earliest lesions was estimated from the description given by the Veterinary Officer examining the animal(s) and from that we determined the period of 4–10 days (i.e. range of incubation period) during which infection of the animals could have occurred.

The primary outbreak and initial spread of virus

Farm 1

Unless airborne virus from the Continent is assumed, which is unlikely (Hurst, 1968), farm 1 could not have been the primary outbreak. The only recorded movement to and from the farm was a meal lorry, which was unlikely to have carried infection. The virus is presumed to have reached the farm by the airborne route. With an incubation period of at least 4 days according to our criteria, a source of airborne virus for farm 1 must have been present in the area on 1 January 1967, if not earlier. Four farms had pigs with lesions described as at least 48 hr. old – 4, 9, 13 and 18 – and one or more of these could be suspected of having produced airborne virus on 1 January 1967.

Farm 4

Farm 1 was not downwind of farm 4 from the early morning of 26 December 1966, until disease was found on 5 January 1967. Farm 4 is therefore unlikely to have been the airborne source of disease at farm 1. On 29 December 1966, and on 3 January 1967, pigs were taken from farm 4 to the abattoir (9) at Fareham.

Fareham abattoir (9)

Farm 1 was downwind of the abattoir (winds 220°–280°) during 29 December. The pigs from farm 4 could have disseminated airborne virus on arrival at the abattoir or could have infected other pigs held at the abattoir which, in turn, could have disseminated virus on 31 December 1966, and 1 January 1967, when farm 1 was still downwind of the abattoir. There was no killing of animals on the afternoon of Saturday, 31 December 1966, and on Sunday, 1 January 1967.

Pigs brought from farm 4 on 3 January could also have had disease but they or pigs housed in the same pens after them were unlikely to have given out airborne

Table 4. *Bearings and distances from infected farms and dates of lesions and incubation periods*

Suggested origin	Infected farm	Bearing to suggested origin	Distance (km.)	Estimated earliest date of lesions	Period of 4- to 10-day incubation period
9	1	267	9	5 January	26 December– 1 January
	2	264	6	5 January	26 December– 1 January
	3	270	1·5	6 January	27 December– 2 January
	5	280	2·5	8 January	29 December– 4 January
	7	303	2	9 January	30 December– 5 January
	8	270	4·5	8 January	29 December– 4 January
	10	268	6	9 January	30 December– 5 January
	11	278	3	8 January	29 December– 4 January
	12	276	10	9 January	30 December– 5 January
	15	079	1·5	10 January	31 December– 6 January
	19	025	3·5	11 January	1 January – 7 January
	21	091	1·5	13 January	3 January – 9 January
	22	084	1	14 January	4 January –10 January
	23	340	0·5	14 January	4 January –10 January
	26	090	1	16 January	6 January –12 January
	25	218	4	16 January	6 January –12 January
13	17	225	0·5	10 January	31 December– 6 January
	25	355	7	16 January	6 January –12 January
	27	297	6	19 January	9 January –15 January
1	12	325	1·5	9 January	30 December– 5 January
10	20	245	1	13 January	3 January – 9 January
16	24	308	2	15 January	5 January –11 January
24	28	090	3	24 January	14 January –20 January

virus to farm 1 because the incubation period was too short (i.e. 2 days). Other outbreaks on farms downwind of the abattoir during the period from 29 December 1966, were at farms 2, 3, 5, 7, 8, 10, 11 and 12 and in Table 4 the bearings, distance, estimated earliest date of lesions and dates covered by a 4–10 day incubation period are shown. Farms 2 and 3 must have been infected on or before 1 and 2 January respectively; the other farms (5, 7, 8, 10, 11 and 12) by 4 or 5 January 1967, though probably not before 29 and 30 December 1966. During the period the abattoir was closed (from 13.00 hr. on 31 December until 05.00 hr. on 2 January), disease could have developed in the pigs held there; this time could have been the optimum for dissemination of airborne virus to all the farms mentioned, as the pigs would be giving out virus at this time and the wind was blowing from 250°–290°. Alternative possible sources of infection by movement of people or vehicles exist for 2, 5, 10 and 11 (Table 2) and the disease at farm 12 could have been airborne either from the abattoir (9) or from farm 1. The abattoir was a potential source of infection until disease was found on Monday, 9 January 1967, i.e. after the weekend (7 and 8 January) during which pigs may well have been disseminating disease (see later).

That infection and disease were present at the abattoir (9) on 2 January 1967, is suggested by the records of visits listed in Table 2:

(i) The owner of farm 5 took calves to the abattoir on 2 and 6 January 1967.

If he took infection back to his premises on 2 January, the incubation period was 6 days. (However, the source of infection is more likely to have been airborne – see before.)

(ii) The owner of farm 15 visited the abattoir·on 2 January 1967, and on his return fed his cattle, which showed lesions on 10 January, i.e. an 8-day interval. Farm 15 was downwind of the abattoir on 7 January 1967; this would give a 3-day incubation period, which according to our criteria is too short.

(iii) Although it is possible that the owner of farm 16 took infection back to his premises after his visit on 2 January 1967, there were two other opportunities – on 4 January when he went among pigs at the abattoir (9) and on 5 January when he delivered and brought back a bullock which subsequently developed lesions. (He also brought back another seven cattle on the same day but they did not have lesions at the time of slaughter.)

(iv) The owner of farm 13 took two pigs to the abattoir on 30 December 1966. He had been collecting slaughterhouse waste daily from the abattoir for pig feed until movement restrictions were imposed after the outbreak at farm 1. Lesions developed in the pigs nearest to where the waste was dumped. At farm 17 the one affected animal probably had lesions on 10 January 1967. Assuming a 4-day incubation period, the infection must have reached the animal on 6 January, i.e. pigs at farm 13 were excreting virus on 6 January, if not earlier. The owner of the pigs at farm 18 probably transferred infection from farm 13 where he worked. The lesions were described as at least 48 hr. old, so that disease was probably apparent on 10 January, suggesting the presence of disease at farm 13 on 6 January. With a 3- or 4-day minimum incubation period for pigs fed on slaughterhouse waste, 2 or 3 January was the latest time that pigs could have received infection from the abattoir waste.

(v) The owner of farm 14 visited the abattoir (9) on 29 December 1966, and on 2 January and 4 January 1967. Disease in one pig developed on 10 January 1967, indicating an 8- or 6-day incubation period.

In summary, (iv) and (v) point to the presence of FMD at the abattoir on 3 and 4 January 1967, and possibly a day earlier. It could also be argued that the abattoir was the primary case and had infection in pigs on 29 December 1966. On that day infection would have been carried to farm 4; carriage on 3 January 1967 would give too short an incubation period, unless the lesions in pigs at farm 4 were not more than 48 hr. old. Infection would also have been carried in the slaughter-house waste to the pigs at farm 13.

Farm 13

The pigs at farm 13 could also have been the primary source of infection. Slaughterhouse waste was collected from the abattoir daily and two pigs were delivered to the abattoir on 30 December 1966. The infection would have had to be transferred to the abattoir (9) before 29 December in order to infect the pigs at farm 4.

Of the three possible primary sources of infection, farm 4 appears to have been the most likely.

Subsequent spread of infection

From farm 4

As well as being a source from which the abattoir apparently became infected, farm 4 was the most likely source of disease for farm 6 which belonged to the same owner. No other spread can be assigned to farm 4, since a possible airborne spread to farm 12 on 7 January 1967 would demand too short an incubation period (2 days).

From Fareham abattoir (9)

From 7 to 9 January the winds were from the north-east quadrant and it is likely that farms 19, 21, 22 and 23 became infected in this period (Table 4). No killing took place from 13.00 hr. on 7 January until 05.00 hr. on 9 January and during this period lesions were probably present or developing. If the cattle at farm 15 were infected by airborne virus, it must have been a 3-day incubation period or at a period of calm or light winds. The cattle at farm 26 could have received infection from the abattoir (9) or from farm 15.

On the night of 6/7 January the wind at Thorney Island backed to the south-west and then veered to the north-east. During this period the cattle at farm 25 could have become infected, giving a 9- or 10-day incubation period (Table 4, see next paragraph).

From farm 13

The infection of the cattle at farm 17 has already been described. The owner of the pigs at farm 18 probably transferred infection from farm 13. The pigs at farm 13 and 18 could have been the source of airborne virus for farm 25, as suitable winds occurred on 9 January, giving an incubation period of 7 days. They could also have been a source of airborne virus for farm 27 on 9–12 January, with an incubation period of 7 to 10 days (Table 4).

From farm 16

Winds on 10 and 11 January could have taken virus to farm 24 (Table 4).

From farm 24

On 15 and 16 January winds could have carried virus to farm 28. Alternatively, the virus came at the time of milk collection (Table 4).

From farm 1

Farm 1 or the abattoir (9) could have been the source of virus for farm 12 (Table 4).

From farm 10

Disease at farm 20 could have come from farm 10 either on 8 or 9 January, giving a 4- or 5-day incubation period (Table 4).

The source of infection for farm 29 is uncertain. The animals may have received infection from farm 23 on 14 and 15 January. There may have been several cycles of disease.

Table 5. *Risks of spread through movement*

	Animals and vehicles	Slaughter-house waste	Milk (farms)	Other vehicles	A.I.	People
Approximate number of movements	72	2	52	54	73	20
Probable movements associated with disease	4 (5·6%)	2 (100%)	0	0	0	1 (5%)
Possible movements associated with disease	2 (2·8%)	0	3 (5·8%)	0	1 (1·4%)	2 (10%)
Probable + possible	6 (8·4%)	2 (100%)	3 (5·8%)	0	1 (1·4%)	3 (15%)

Risks of spread through movement

In Table 5 is shown the number of movements to and from the farms in the area recorded by the Veterinary Officers investigating the outbreak. An estimate of the risk involved has been made by ascribing 'probable' or 'possible' to those movements that were involved in subsequent disease on the farms. Of the movements probably associated with disease, movement of slaughterhouse waste, movements of animals in vehicles and movements of people were the most likely.

Airborne spread of disease

We have ascribed thirteen outbreaks (with the possible addition of another three) to airborne spread from Fareham abattoir (9), three to airborne spread from farm 13 and none to airborne spread from farm 4. According to the records, ten pigs with lesions were found at Fareham abattoir, 126 at farm 13 and 141 at farm 4. If the number of pigs emitting virus was the only factor, a greater number of outbreaks would have been expected around farms 13 and 4. In a previous paragraph we gave reasons for considering that pigs at the abattoir were emitting virus from 29 December until 9 January but we cannot estimate the number of pigs or the amount of virus. During the same period until 8 January, pigs at farm 4 were also infected and probably emitting virus.

At farm 4 the pigs were housed in sheds situated on the north-eastern side of a hill and screened on the south by trees. Most of the area around farm 4 is town and the number of cattle within a 3-mile radius was less than half of that around the abattoir (Table 6). The winds during the period were from 250° to 360° and the virus would have been blown over built-up areas or out to sea. The turbulence over the built-up area would tend to disperse the virus and reduce the concentration. When the winds were from 360° to 120° on 7 January or after, the dispersal of virus would again have been over built-up areas.

Of the area around the Fareham abattoir (9), the sector to which winds from bearings 250°–290° blew had the greatest number of farms with infected cattle and the furthest spread. In addition, the number of cattle per 10° was the highest (Table 6). The abattoir is in the valley and winds from 290° to 360° (3 to 6 January) would have blown the virus over Fareham.

Table 6. *Numbers of cattle per 10° arc within a 3 mile radius*

		Farm		
		4	9	13
Total number of cattle within a 3 mile radius		1580	3727	3321
Number of cattle per 10° arc of wind bearings	360°–120°	39	110	124
	120°–180°	55	40	64
	180°–250°	15	104	45
	250°–290°	125	179	77
	290°–360°	25	32	119

The remaining infected farms around Fareham lie to the south-west of the abattoir and from 7 January to 9 January winds blew from 10° to 70°. To the north-west of the abattoir (winds 120°–180°) is a wood but north-east the number of cattle per 10° is similar to that to the south-west and the finding of only one outbreak in this area could be attributed to the fact that winds from the south-west were infrequent during the period when pigs were infected.

The number of cattle within a 3-mile radius of farm 13 was similar to those within three miles of Fareham abattoir (Table 6) but only three outbreaks can be attributed to airborne spread. This probably is because emission of virus from the pigs did not start until 5 January and lasted to 10 January, i.e. a shorter period of emission. In addition, the piggery was protected from the action of the wind by being situated in a court-yard on the southern slope of a hill and being screened by trees.

In summary, therefore, the differences in extent of airborne spread between the three premises can be attributed to period of virus emission (abattoir 9 and farm 13), number of cattle and extent of built-up areas downwind (abattoir 9 and farm 4) and situation of the infected farms (9, 4 and 13).

In laboratory experiments, cattle have been shown to give rise to less airborne virus than pigs (Sellers & Parker, 1969) and, in the field, outbreaks in cattle would not be expected to give rise to as many subsequent outbreaks by the airborne route. In our analysis three secondary outbreaks were attributed to spread from cattle (i.e. outbreaks at farms 20, 24 and 28), with the possibility of another three (at farms 12, 22 and 26). In the probable outbreaks, spread was to one farm only; of the possibles, both outbreaks at farms 22 and 26 could have come from farm 15.

Where spread could be attributed to the airborne route, the mean size of the herd of cattle affected was in all instances higher than the size of those not affected. Where a single herd in a sector was affected, in three instances it was the largest herd and in the other two among the largest (Table 7).

In Table 8 the rainfall and wind speed during the days when airborne spread of virus occurred are given. Relative humidity was above 60 % during the period and airborne virus would not have been inactivated (Barlow, 1972; Donaldson, 1972). From the table, spread could have occurred during periods with or without rain. During the two periods when spread was most likely from Fareham abattoir (9), on 31 December and 1 January, 40 hr. of the 48 were without rain and, out of

Table 7. *Number of farms and of cattle on farms downwind*

Source	Bearing of wind	Distance downwind	Number of farms not affected	Cattle	Number of farms affected	Cattle
Abattoir (9)	250°–290°	0–3 miles	4	34, 48, 52, 79 Mean = 53·25	5	45, 66, 93, 130, 139 Mean = 94·6
		3–6 miles	22	2, 14, 16, 27, 28, 29, 31, 38, 38, 41, 48, 60, 61, 65, 65, 81, 86, 92, 95, 104, 116, 118 Mean = 55·36	5	84, 86, 106, 123, 128 Mean = 104·8
	360°–120°	0–1·5 miles	5	1, 4, 19, 33, 65 Mean = 24·40	4	14, 49, 69, 94 Mean = 56·60
		1·5–3·0 miles	24	1, 1, 2, 3, 4, 4, 5, 6, 7, 8, 10, 11, 13, 14, 14, 16, 23, 26, 53, 78, 81, 97, 193, 209 Mean = 32·46	1	97
	180°–250°	0–3 miles	12	1, 1, 4, 5, 10, 12, 24, 77, 104, 116, 118, 178 Mean = 54·17	1	69
Farm 13	180°–250°	0–3 miles	14	2, 3, 3, 7, 8, 8, 11, 13, 17, 22, 24, 26, 37, 47 Mean = 16·29	1	76
	290°–360°	0–3 miles	21	2, 4, 4, 8, 8, 8, 10, 15, 16, 18, 28, 28, 29, 34, 39, 39, 41, 41, 58, 76, 85 Mean = 26·24	2	69, 236 Mean = 152·5
Farm 16	250°–360°	0–2 miles	13	3, 5, 6, 6, 11, 25, 27, 38, 44, 64, 65, 74, 95 Mean = 35·6	1	134
Farm 24	70°–170°	0–2 miles	19	3, 5, 6, 7, 8, 12, 15, 19, 19, 21, 22, 25, 26, 36, 36, 42, 67, 69, 95 Mean = 28·05	1	98

Table 8. *Rain and wind speed during possible days of spread during the epidemic*

Date	Possible sources and farms downwind	Duration (hr.) and amount of rain (mm.)	Time	Hours of wind ≥5 knots	≥10 knots
29 Dec.	9 → 1, 2, 3, 5, 8, 11	10 (10·95)	01.00–11.00	24	23
30 Dec.	9 → 1, 2, 3, 5, 8, 10, 11, 12				
	(10 hr.)	—	—	6	4
	(14 hr.)	4 (0·75)	21.00	13	6
31 Dec.	9 → 1, 2, 3, 5, 8, 10, 11, 12	8 (3·0)	to 10.00	24	22
1 Jan.	9 → 1, 2, 3, 5, 8, 10, 11, 12	—	—	24	14
2 Jan.	9 → 5, 7	—	—	20	3
3 Jan.	9 → 5, 7	—	—	22	2
4 Jan.	9 → 5, 7	—	—	23	0
5 Jan.	9 → 7				
	1 → 12	—	—	18	11
6 Jan.	1 → 12 (16 hr.)	—	—	13	3
	9 → 25 (8 hr.) 13 → 17 (8 hr.)	8 (0·15 snow) (2·8 rain)	17.00 to midnight	8	2
7 Jan.	9 → 25 (6 hr.) 13 → 17 (6 hr.)	5 (2·3)	01.00–05.00	6	3
	9 → 19, 21, 22, 23, 26 (18 hr.)	5 (0·5)	07.00–09.00 14.00–15.00	18	16
8 Jan.	9 → 21, 22, 23, 26 10 → 20	7 (trace) (traces)	10.00 17.00–23.00	24	19
9 Jan.	9 → 21, 22, 23, 26 13 → 25, 27 10 → 20 16 → 24	—	—	17	8
10 Jan.	13 → 27 16 → 24	3 (trace)	14.00–15.00 19.00	12	0
11 Jan.	18 → 27 16 → 24	—	—	14	0
12 Jan.	18 → 27	—	—	21	0
15 Jan.	24 → 28	—	—	6	0
16 Jan.	24 → 28	—	—	16	3

50 hr. on 7, 8 and 9 January, 12 had light rain only. A notable feature during these periods was the number of hours when the wind speed was 10 knots or greater.

In Table 9 we have listed the outbreaks apart from farms 4, 9, 13, 18, 1, 10, 15, 16 and 24 together, where appropriate, with reasons to explain subsequent lack of spread apart from insufficient output. In the majority of cases the virus would have been blown over built-up areas, woods or land where the density of livestock was low. We can offer no explanation for three of the outbreaks (farms 17, 27 and 28), although at farm 17 only one cow had disease.

DISCUSSION

Any analysis of this epidemic must be speculative because the evidence is circumstantial. Our analysis differs from that of others (Report on the Animal Health Services in Great Britain, 1967; Smith & Hugh-Jones, 1969) in assuming that infectious virus was present in the area on 1 January 1967, if not before then. We cannot otherwise account for the first reported outbreak (farm 1) or for the

Table 9. *Possible reasons for failure of airborne virus to spread*

Farm	No. of animals affected	Bearing of winds at time of disease	Reasons for lack of subsequent outbreaks
2	5	290°–360°	Wind to Portsdown
3	21	190°–120°	Surrounded by infected farms
5	2	10°–80°	Wind to Portsdown
7, 8, 11	4, 12, 4	330°–80°	Wind to Portsdown
12	22	270°–20°	Wind to Portsmouth and Havant
19	4	250°–330°	Wind to Gosport and Fareham
20	4	270°–330°	Wind to Portsmouth
21, 22, 23	2, 2, 4	250°–360°	Wind to Fareham
25	21	80°–210°	Wind to woods and town
26	1	80°–210°	Wind to woods
29	10	230°–290°	Surrounded by built-up areas or previously infected farms
6	4	330°–50°	Wind to Hayling Island, Portsmouth and sea
14	1	250°–330°	Surrounded by woods
17	1	250°–330°	Unknown
27	10	150°–210°	Unknown
28	8	200°–290°	Unknown

next one or two outbreaks. As mentioned in the paragraph on criteria, we postulate a minimum dissemination period between farms of 3 days for pigs and 4 days for cattle. Applying these criteria to the recorded infections, we have worked backwards and forwards in time to fill in details of a pattern of spread that might have taken place.

All evidence points to the dissemination of FMD from the abattoir. We suggest that infection was present at the abattoir from 29 December and one may wonder why disease was not reported until 9 January, when lesions described as at least 2 days old were discovered. Lesions of foot-and-mouth disease in pigs due to the O_1 strain are difficult for the inexperienced to detect. Four of the other five outbreaks where the lesions reported were over 48 hr. old were in pigs and, in the outbreaks at Stratford-on-Avon and Oswestry in 1967, lesions in pigs were reported to be 7 to 10 days and 4 days old, respectively (Report on the Animal Health Services in Great Britain, 1967). Until 7 January 1967, pigs were probably being killed at the abattoir in the early stages of disease when output of airborne virus was maximal (Sellers & Parker, 1969; Donaldson *et al.* 1970); after processing, the lesions would be difficult to detect and the carcase would show no sign of fever. During the period from 29 December to 9 January, there were two weekends when animals were held alive and this break in slaughtering was probably important in allowing spread of disease among the pigs at the abattoir and to the surrounding countryside.

In this epidemic two main methods of virus spread are apparent – one by

movement of animals, slaughterhouse waste (swill), vehicles and people, and the other airborne. Analysis of the movements leading to probable infection showed that movement and feeding of slaughterhouse waste, movement of animals and vehicles and movement of people were the most important. How this occurs has been discussed elsewhere (Sellers, 1971). Where airborne spread and spread by movement were both possibilities (farms 2, 5, 11 and 15), we have considered airborne spread more likely in three instances (farms 2, 5, 11), since winds were available, and less likely in the fourth (farm 15), since the incubation period would have been too short. Spread by artificial insemination, milk lorries or wild animals or birds was also considered less likely because the amounts of virus available would have been less than from infected pigs (Sellers, 1971).

Where spread was attributed to the airborne route, virus coming from pigs gave rise to a greater number of outbreaks than virus from cattle. When calculations were made with Pasquill's (1961) formula, the amount given out by pigs ($10^{4 \cdot 8}$ ID 50 per pig per minute – Sellers, 1971) was sufficient to account for the cattle downwind breathing in an infective dose (10 ID 50) within a reasonable period. However, the calculated downwind concentration of virus based on the amount emitted by cattle was insufficient to account for infection from farm 10 to farm 20, farm 16 to farm 24 and farm 24 to farm 28. Possibly in these cases spread was not airborne or was from faeces (Parker, 1971) or the virus was not diluted to the extent predicted by the formula.

The greatest extent of airborne spread was in the areas of high cattle density but dispersion of virus was also affected by the situation of the infective farms and the duration of virus emission. The largest herds were the ones most likely to be infected downwind and, if this is confirmed from studies of other outbreaks, it would be advisable to look for disease in such animals when attempting to trace spread of disease.

In our analysis, most airborne spread occurred during the period when wind speeds of 10 knots or over were recorded. If the larger particles ($> 6\ \mu$m) are the more important in initiating infection (Sellers & Herniman, 1972), strong winds would blow them upwards, keep them airborne and prevent deposition under sedimentation. When rain occurred during the period when spread was likely, on 31 December and 7 January, it was light (trace to 0·9 mm. per hr.) and thus would be unlikely to cause deposition of the virus (Sellers & Herniman, 1972).

The decline in the number of outbreaks may be attributed to a number of factors but mainly to the elimination of the pigs as a source of virus. After 12 January only cattle were a source and, as previously mentioned, they emitted less virus. In addition to the restrictions which were in force, the winds were blowing the virus into built-up areas, into woods or to farms where the herds had been eliminated. However, in three instances no explanation is offered for failure to spread, apart from insufficient amounts of virus.

Our analysis therefore falls between that of the Report (1967) and that of Smith & Hugh-Jones (1969), the one attributing most spread to movement and contiguity, the other to wind spread during rain. Our analysis and experimental work (Sellers & Herniman, 1972) indicate that rain may not be such an important factor

as originally thought, although wind speed may play a part (Tinline, 1969; Hugh-Jones & Wright, 1970). However, the meteorological evidence for the wind-rain sector of 160° on the night of 6/7 January is not clear (Smith & Hugh-Jones, 1969). It snowed and rained but the surface winds at Calshot and Thorney Island veered from south-west to north-east, whereas at Southampton and South Farnborough there was a period of calm between winds from the south-east and winds from the north-east.

The fact that the first reported outbreak was not the primary and that the primary would not have been apparent until 8 or 9 January would have made it difficult in the Hampshire epidemic to predict at the time which way spread would occur. Movement of animals, swill, people and vehicles was still continuing until 5 January and it was not until after 12 January, 7 days later (an average incubation period), that outbreaks could be attributed to spread by the airborne route alone. Thus, in any prognosis of the outcome of an epidemic, the main factors involved in spread such as movement (controllable) and airborne (uncontrollable) must be taken into account during the first incubation period.

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Antibody to streptococcal opacity factor in human sera

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SUMMARY

Two tests are described for detecting antibody to the type-specific opacity factor (OF) of group A streptococci. This antibody was detected among patients convalescent from streptococcal sore throat in two communities in which outbreaks due to opacity factor-producing strains of group A streptococci occurred.

In an outbreak due to streptococci of M-type 22 there was a close correspondence between the distribution of anti-OF and of bactericidal M-antibody for the type. In a smaller outbreak due to M-type 58 streptococci, however, M-antibody was detected more often than antibody to OF.

INTRODUCTION

The streptococcal opacity factor (OF) is found in all members of 16 clearly differentiated M-types of group A streptococci and in a number of other strains in which the M-antigen has not yet been identified. It causes the appearance of opacity in horse serum (Ward & Rudd, 1938; Gooder, 1961) and antibody to it inhibits this opacity (Top & Wannamaker, 1968). The antigenic specificity of OF runs parallel with that of the M-antigen (Widdowson, Maxted & Grant, 1970), so that inhibition is observed only when OF is mixed with antisera against a member of the same M-type. The OF is closely associated with the M-antigen in the bacterial cell and the two may form part of the same complex protein (Widdowson, Maxted, Grant & Pinney, 1971).

Before the type specificity of the OF had been established, Krumweide (1954) reported the presence of antibody to it in human serum. However, no attempt to study the occurrence of the antibody in serum of patients with streptococcal disease has since been reported. The type specificity of the opacity factor and its undoubted close association with M-antigen suggested that the identification of antibody to OF might also be an indication of the presence of protective antibody to the M-antigen.

In the course of other work (Maxted *et al.* 1973) we obtained active preparations of the OF of most of the known opacity producing M-types and made rabbit antisera against some of these opacity factors. We were thus in a position to investigate possible tests for antibody against OF and then to test sera from patients who had recovered from streptococcal disease for antibody to the infecting type.

Two outbreaks of streptococcal infection of the upper respiratory tract, one due to M-type 22 and the other to M-type 58, provided the sera used in the investigation reported here. Both of these serotypes are OF positive.

MATERIALS AND METHODS

Preparation of opacity factor

Streptococci of each known OF-positive M type (types 2, 4, 9, 11, 13, 22, 25, 28, 48, 49, 58, 59, 60, 61, 62, 63 and provisional types 67/3875, 453, 3354, PS346, PS432) were grown overnight in Oxoid Todd Hewitt Broth to which 1 % (w/v) Neopeptone had been added. The supernatant after centrifugation at 3500 g was tested for its activity against horse serum (see below); if this was satisfactory, thiomersal 1/5000 (w/v) was added and the supernatant kept at $-20°$ C.

Two tests were used to establish the OF activity of each supernatant.

The tube test was done by incubating 0·2 ml. of supernatant with 1·0 ml. horse serum (Recalcified Plasma No. 2. Wellcome Research Laboratories, Beckenham, Kent) containing thiomersal 1/5000 (w/v) for 18 hr. at 37° C. Physiological saline 1·2 ml. was added to the tubes and the optical density (A_{475}) was read on a Unicam SP 600 spectrophotometer. A control tube containing 1·4 ml. saline and 1·0 ml. horse serum was included. An increase in the opacity of 0·1 or more over the control tube was taken as a positive reaction; strongly positive supernatants gave an increase of at least 0·2–0·3.

Serum-agar slides. Equal volumes of 2 % Ion Agar (Oxoid) and horse serum were mixed, and 6 ml. of the mixture was poured onto 2 in. × 2 in. (5 cm. × 5 cm.) glass slides. The surface moisture was dried off by incubating the slides at 37° C. for 20 min. Supernatants were spotted on the slides with a 2-mm. diameter wire loop, and the slides incubated in a moist chamber overnight. Zones of opacity, varying in intensity with the OF activity of the supernatant, were seen in the area of application of the supernatant.

Sera

Preparation of anti-OF serum

These were prepared in rabbits. Three were sera made by injecting cell-wall fragments of streptococci, of M-types 2, 4 and 25 respectively (Widdowson *et al.* 1970). Other sera were unabsorbed M-typing sera, made in the conventional manner in the Streptococcus Reference Laboratory (Williams, 1958) and found to contain OF antibody that inhibited the serum opacity reaction of the homologous strain. To ensure that the antisera were specific, each serum was tested against the OF of several streptococci of the homologous type, a large number of OF positive strains with identical T-agglutination patterns but without identifiable M-antigen, and a selection of other strains that were quite unrelated.

Patient's serum

Sera were collected about 4 weeks after infection, and stored at $-20°$ C immediately upon receipt in the laboratory.

Typing sera and typing methods

The methods used in the Streptococcus Reference Laboratory for the preparation of typing sera and for M & T typing were described by Williams (1958).

Specific inhibition of the serum opacity reaction

Tube method

Two-fold dilutions of anti-OF serum were made in physiological saline. To 0·02 ml. volumes of each dilution was added 0·1 ml. of the culture supernatant of an OF-positive streptococcus diluted to five times the concentration necessary to cause opacity. The tubes were shaken and incubated at 37° C. for 30 min.; 1 ml. of horse serum was added to each tube, the tubes again shaken and incubated overnight. A tube of horse serum without antiserum and another of horse serum without OF were always included as controls and the opacity was estimated visually or after dilution with an equal volume of saline. The A_{475} was read spectrophotometrically (Widdowson *et al.* 1971).

To identify the OF of a streptococcus, 0·02 ml. of each available anti-OF serum, in a dilution previously shown to inhibit the OF of the homologous strain, was added to a tube containing 0·2 ml. of the supernatant and 1 ml. of horse serum.

Solid agar method

Equal volumes of 2 % Ion Agar and horse serum were mixed; to 6 ml. of the mixture, 1·2 ml. of diluted active OF supernatant was added and after thorough mixing poured on a 2 in. × 2 in. glass slide. A loopful (2 mm. diameter) of each antiserum, undiluted or diluted for titration, was placed on the dried agar surface. The slides were incubated overnight in a moist chamber. The reaction of the OF with the horse serum produced an opaque background against which clear areas of inhibition were seen.

The indirect bactericidal test

The bactericidal power of normal heparinized human blood for streptococci is enhanced by the addition of human serum containing M-antibody of the homologous streptococcus. The test was done by adding 0·02 ml. of a suitably diluted broth culture (50–500 viable units) to 0·02 ml. of patient's serum (undiluted, or after diluting 1/5 or 1/10 in an attempt to get a more quantitative estimate of the antibody present. A 0·3 ml. volume of heparinized blood from a donor previously shown to have no antibody to the test strain was then added to each tube. The tubes were sealed and rotated end-over-end at 37° C. for $3\frac{1}{2}$ hr. and an estimate of the viable streptococci present made, by subculturing 0·02 ml. volumes of the mixture into molten blood agar, pouring blood agar plates and counting the colonies after 18 hr. incubation. The growth was recorded as:

$$- \; = \; 0 \text{ to } 9 \text{ colonies,}$$
$$+ \; = \; 10\text{--}49 \text{ colonies,}$$
$$+ + \; = \; 50\text{--}249 \text{ colonies,}$$
$$+ + + \; = \; 250\text{--}999 \text{ colonies,}$$
$$+ + + + \; = \; \text{discrete but uncountable number of colonies.}$$

Table 1. *Presence of antibody to type 22 OF in human sera; 67 sera were tested by the tube and the slide method*

Serum no.	Depression of A_{475}	Slide reaction
377	0·22	+
378	0·25	+
387	0·28	+
389	0·32	+
390	0·33	+
395	0·32	+
396	0·21	+
398	0·28	+
403	0·35	+
421	0·33	+
434	0·25	+
436	0·26	+
54 other sera	0	−

+ = inhibition of serum opacity reaction.
− = no inhibition of serum opacity reaction.

RESULTS

Rabbit anti-OF sera to streptococcal OF

Active OF supernatants and corresponding antisera were available for M-types 2, 4, 9, 28, 48, 49, 58, 59, 62 and 63 and for four further provisional types (67/3875, 453, PS346, PS432). Each supernatant was tested for inhibition by each antiserum; in every case inhibition of opacity was observed with the antisera of the homologous M type and not with the heterologous antisera, with the single exception that the OF of type 9 was inhibited not only by the type 9 antiserum but to some extent also by the type 28 antiserum. The non-specific inhibition shown by this serum could be removed by absorbing the type 9 OF antiserum with type 28 cells. The type 9 OF-antiserum was then specific and inhibited type 9 OF only.

The sera were also tested for inhibition of the OF of a number of strains of the homologous type, and of a variety of strains with similar T agglutination patterns but with an unidentifiable M-antigen. Each opacity factor was inhibited specifically by a single antiserum.

Antibody to opacity factor in human sera

Sera from an outbreak of M-type 22 infection

In the spring of 1970, there was a large outbreak of streptococcal sore throat in a boys' school. Bacteriological investigations were carried out late in the outbreak, and it appeared that two types of streptococcus were predominant; both had the T-typing pattern 12, and one had M-antigen 22 and the other the M-antigen 12. The former was OF positive and the latter OF negative. Cultures were received from 16 boys, of whom 11 yielded the type 22 streptococci and 5 the type 12 streptococci. Sera were collected from 67 boys about 4 weeks after the peak of the outbreak.

Table 2. *Titration of antibody to OF in patients' sera.*
Comparison of the tube and agar slide methods

	Serum no. 378			Serum no. 387			Serum no. 421	
Diluted	A_{475} depression	Slide reaction	Diluted	A_{475} depression	Slide reaction	Diluted	A_{475} depression	Slide reaction
1/5	0·21	+ + +	1/5	0·15	+	1/5	0·21	+ + +
1/10	0·22	+ +	1/10	0·10	tr	1/10	0·22	+ +
1/20	0·23	+	1/20	0	−	1/20	0·23	+ +
1/40	0·21	+	1/40	0	−	1/40	0·21	+
1/80	0·15	±	1/80	0	−	1/80	0·16	+
1/160	0·05	tr	1/160	0	−	1/160	0·1	±
1/320	0	−	1/320	...	−	1/320	...	tr
1/640	0	−	1/640	...	−	1/640	0	−

+ + +, + +, +, ±, tr = strength of inhibition of serum opacity reaction on an arbitrary scale.

− = no inhibition of serum opacity reaction.

... = not done.

The results of the initial screening of the sera for antibody to OF alone, done by the two methods, appear in Table 1. The optical density readings and agar slide results agreed well. It was found that horse serum + opacity factor + normal human serum, gave a higher reading (0·75) than OF and horse serum alone (0·6). It is apparent that a control mixture containing a human serum known to be devoid of OF antibody must be included in every batch of tests.

There was considerable variation in the strength of the OF inhibition, seen with the patients' serum (Plate 1). Three of these, each differing in the strength of the initial reaction, were diluted and the OF activity titrated on slides and in tubes. The two methods gave good agreement and the end points reflected the degree of activity seen in the initial test with the undiluted sera. In the tube test a difference of 0·1 in the A_{475} reading was taken as evidence of inhibition and this difference could be detected on the serum agar slides also (Table 2).

All the sera were treated similarly, and in Table 3 a quantitative estimate of the anti-OF titres are given together with the result of the bactericidal tests.

In the bactericidal test, 15 sera showed the presence of M-antibody for type 22 (22 % of the sera tested). Antibody to OF was detected in 12 of these 15 sera and in none of the remaining 52 sera.

The results of the bactericidal tests were derived from four separate experiments. Tests using either graded doses of patient's serum or graded streptococcal inocula did not always show the expected gradation of the killing effect. However, the sera could be classified semi-quantitatively into those that gave good, moderate, poor or no killing effect based on the growth seen under the conditions of the experiment as indicated in the footnote to Table 3.

The sera that consistently gave the best bactericidal effect when added to whole blood also had the highest antibody titres (1/640) against the OF of type 22. Similarly, 2 of the 3 sera that were negative in the anti-OF test were the weakest of the 15 sera that showed any bactericidal power at all.

Table 3. *Patients' sera: comparison of OF antibody titre with bactericidal activity against M type 22 streptococci*

Serum no.	Bactericidal activity against M type 22*	OF antibody titre†
389	Good	> 640
378	Good	640
390	Good	640
421	Moderate	320
395	Moderate	80–160
434	Moderate	80–160
396	Moderate	20
380	Moderate	0
377	Poor	80–160
398	Poor	160
403	Poor	20
387	Poor	10
436	Poor	10
406	Poor	0
417	Poor	0
12 other sera fully tested	Nil	0
40 sera screened	Nil	0

* Good = 0·02 ml. of a 1/5 dilution of the serum reduced + + + + to < + + survival in the bactericidal test (see Methods).
Moderate = 0·02 ml. of the serum reduced + + + + to < + + survival but 0·02 ml. of a 1/5 dilution had no effect.
Poor = 0·02 ml. of the serum reduced + + + + to + + or + + + survival.
Nil = 0·02 ml. of the serum had no effect on survival.
† = Reciprocal dilution in the tube test.

All the sera were also tested for bactericidal activity against M type 12 and this was found in 32 sera (47·7 %).

Sera from M-type 58 infections

In 1971, an outbreak of acute upper-respiratory tract infection occurred in a training centre for RAF cadets, in which it appeared that three M-types of strep-tococci were involved: type 5, type 18 and type 58. Type 5 and type 18 are OF negative but type 58 is OF positive, and attempts were made to find type 58 OF antibody in the serum of the patients by the same two methods as before.

Bactericidal tests were done on 142 sera and M-type specific antibody to M58 was found in four of them, but only one of these four had detectable antibody to type 58 OF (titre < 1/10). None of the 138 sera in which M58 antibody was not detected had antibody to type 58 OF. Bactericidal antibody to M-type 5 was found in 36 sera and to M-type 18 in 18 sera.

DISCUSSION

This investigation is the first to report the presence of antibody to OF in the serum of patients recovering from infection with a streptococcus of a known M-type.

Two points appear to be of importance, the first concerns the antibody as a monitoring marker for the patients' response to the M-type specific antigen and the second the role the OF antibody itself might play in protection of the host. Consideration of these two possibilities is stimulated by the undoubted close relationship between OF and the type specific determinant of M protein. The two have so far resisted separation by physical or chemical means, and their antigenic specificity shows a strict parallelism.

Whole organisms, or fragmented cell-wall material, were used to produce antibody to OF in rabbits, and these contain both OF and M antigen. Many rabbit antisera prepared against OF-positive types – for example, some of the M-typing sera made in the Streptococcus Reference Laboratory – contain M antibody but show no activity when tested for inhibition of opacity produced by the vaccine strain. On the other hand, all sera with anti-OF activity showed some bactericidal effect, though some of them gave a poor tube-precipitation reaction with homologous antiserum.

In the patients' sera, 12 of the 15 in which type 22 M-antibody could be demonstrated by the bactericidal test also contained the corresponding anti-OF antibody. Two of the three sera in which anti-OF antibody could not be detected gave a weak bactericidal reaction. It may be that the poor response to either antigen is related to the severity of infection. Because of the very close relationship of the OF antigen with the type specific determinant, antibody to both might be expected and this was so in 80 % of the sera with type 22 M-antibody, but in only one of four sera with type 58 M-antibody. This may be a consequence of the rather feeble extracellular OF activity sometimes shown by this M-type, compared to the vigorous activity seen with type 22 strains.

The tests we used for detecting OF antibody seem to be precise and sensitive, and identified the antibody in sera showing a considerable variation in anti-OF titres. Although OF is limited in its distribution among streptococcal serotypes, such an additional sensitive marker for antibody to a part of the M protein may be of some advantage when investigating infections with OF-producing types. They are all types in which the M-antibody response is notoriously poor, a characteristic of many of the strains found in streptococcal pyoderma (Wannamaker, 1970).

Antibody to the type specific M-antigen persists for many years (Lancefield, 1959) and is of significance in protection against reinfection. It is of obvious interest to know whether OF antibody has a similar activity and whether it is also persistent.

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EXPLANATION OF PLATE

Inhibition of the opacity factor (OF) of type 22 by 11 of 12 patients' sera on agar containing type 22 OF and horse serum viewed against a black background. (× 1.)
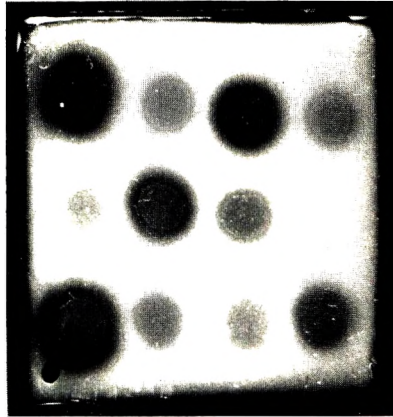
Carrier rate of salmonellas in sheep and goats and its public health significance

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SUMMARY

To find out the salmonella carrier rate, 5980 samples comprising faeces, mesenteric lymph nodes, liver and spleen were collected from 812 sheep and 683 goats slaughtered for food. In all 72 salmonella strains from 51 animals (25 sheep and 26 goats) were isolated. These represented 22 salmonella serotypes. The public health significance of these findings is discussed.

INTRODUCTION

The dissemination of the members of genus *Salmonella* from infected carcasses is a proven route of human infection (Thomsett, 1963). Surprisingly there are few reports from India on the carrier-rate of salmonellas in animals used for human consumption (Kumar, 1964). The present study was undertaken to define the carrier-rate of salmonellas in sheep and goats, slaughtered for food, at Mhow, Central India.

As salmonellosis is primarily an enteric infection and faeces are often responsible for contamination of other carcasses in unhygienic abattoirs, an examination of faecal samples was undertaken. During their course of spread from the intestines to other parts of the body, salmonellas may be trapped in mesenteric lymph nodes, liver and spleen and therefore these organs were also examined in this study.

MATERIALS AND METHODS

Collection of specimens

Faeces, mesenteric lymph nodes, liver and spleen were cultured from each of 812 sheep and 683 goats. Soon after slaughter, 1–2 g. of faeces was collected from the distal part of the large intestine. Approximately 1 g. each of liver and spleen was collected aseptically in separate sterile test tubes. Three to five lymph nodes draining the small and large intestines were collected.

Culture of specimens

Within 1 hr. of collection the specimens were brought to the laboratory and 10 ml. of tetrathionate broth was added. Lymph nodes, livers and spleens were cut into small pieces before adding the enrichment medium. Tubes were incubated for

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Table 1. *Source of* Salmonella *serotypes isolated from 25 sheep and 26 goats*

Salmonella serotype	Sheep*				Goats			
	Faeces	Lymph node	Liver	Spleen	Faeces	Lymph node	Liver	Spleen
S. aberdeen	−	+	−	−				
S. adelaide	−	+	−	−	−	+	−	−
S. anatum	+	−	−	−	+	+	−	−
	+	−	−	−	+	+	+	+
	+	−	−	−	+	+	−	−
S. bareilly	+	−	−	−				
	+	−	−	−				
S. bovismorbificans	−	+	+	−	+	+	−	−
S. chester	+	−	−	−	+	−	−	−
					+	−	−	−
S. choleraesuis	+	−	−	−				
S. derby	−	+	−	−	+	−	−	−
	−	+	−	−	+	+	−	−
S. dublin	−	+	−	−	+	−	−	−
S. enteritidis	+	−	−	−				
S. fremantle	+	−	−	−				
S. frintrop	−	+	−	−	+	−	−	−
S. london					−	+	−	−
S. oranienburg					+	−	−	−
S. poona					−	+	−	−
S. pullorum	−	+	−	−	+	−	−	−
S. reading	+	+	−	−	+	+	+	−
	+	+	+	−				
S. rostock					+	+	−	−
S. salford					−	+	−	−
S. typhimurium	+	−	−	+	+	+	−	−
	+	−	−	−	+	+	−	−
	+	−	−	−	−	−	+	−
					−	+	−	−
					+	+	+	+
					−	−	+	−
S. virchow					−	+	−	−
S. welterveden	+	−	−	−	+	−	−	−
	+	−	−	−				
Total no. of strains	16	10	2	1	18	16	5	2

* One faecal sample from a sheep, not included in this table, yielded S. anatum and S. dublin.

30–36 hr. and then plated on MacConkey agar, Salmonella Shigella (S.S.) agar and Brilliant Green (B.G.) agar (Hormaeche & Peluffo, 1959). Suspect salmonella colonies were inoculated in Triple Sugar Iron (T.S.I.) agar. The T.S.I. tubes showing no change or the production of acid were discarded, while the rest were transferred to urease medium. Urease positive cultures were discarded. Urease negative cultures were further tested for indole production and fermentation of the following sugars: arabinose, xylose, glucose, adonitol, dulcitol, sorbitol, mannitol, salicin and inositol. Other biochemical tests performed were nitrate reduction, Voges-Proskauer, gelatin liquifaction, H_2S production, citrate utilization and growth in KCN medium.

Table 2. *Frequency of isolation of salmonellas from sheep and goats over a seven months period*

Month	No. of animals examined/ No. positive	
	Sheep	Goats
July	121/5	102/6
August	142/6	112/7
September	147/6	85/4
October	136/3	82/6
November	109/2	105/3
December	103/2	112/0
January	54/1	85/0
Total	812/25	683/26

Serological typing

Serological typing of all strains suspected of being *Salmonella* was done by one of the authors (S.K.) at the National Salmonella and Escherichia Centre, India, according to the centre's procedure (Agarwal, 1963).

RESULTS

Twenty-five of the 812 sheep (3·1 %) and 26 of the 683 goats (3·8 %) were found to be salmonella carriers. The total number of strains isolated from all four sources, faeces, liver, spleen and mesenteric lymph nodes was 72, which represented 22 different salmonella serotypes (Table 1).

The differences between the number of positive isolations from male and female animals of both species were statistically not significant.

The apparent higher number of isolations during the warmer months (Table 2), compared to the colder months of the year was statistically insignificant.

The number of isolations was higher from older than from younger animals.

DISCUSSION

The present study revealed that 3·1 % of sheep and 3·8 % of goats were carrying salmonellas. These findings are similar to those of Zwart (1962) in Ghana and Sharma & Singh (1961) in India but differed from workers in England and America who failed to demonstrate any salmonellas in sheep (Smith & Buxton, 1951; Mann, 1963). *S. typhimurium* was the commonest of all the salmonella serotypes recorded in the present study; its frequency of isolation was higher in goats (6) than in sheep (3).

The higher number of isolations from older animals, compared to younger animals, irrespective of species and sex, confirm earlier reports (Edwards, Bruner & Moran, 1948; Buxton, 1957; Salisbury, 1958; Moore, Rothenbacher, Bennett & Barner, 1962). In two young goats, salmonellas were recovered from all the four samples collected from each animal. These results extend the previous findings (Buxton, 1957; Edwards *et al.* 1948) that septicaemic infections are more frequent

in younger animals – the so-called 'doctrine of Montevideo' (Vaccaro, Perez & Fincheira, 1945).

On one occasion, two salmonella serotypes (*S. anatum* and *S. dublin*), were recovered from a single faecal sample of a sheep. Such multiple infections have been observed both in man (Juenkar 1945; Gulasekharam, Velaudapillai & Sabanathan, 1961) and in animals (Edwards *et al.* 1948; Buxton, 1957).

The isolations, especially of salmonella serotypes that have been incriminated in human food poisoning, are of great public health importance as illness due to these organisms is very common in India (Agarwal, 1963). Infected carcasses may contaminate other carcasses or animals during the dressing operations, in transportation or at the butcher's shop, etc. (Camps, 1947; McDonagh & Smith, 1958). Thus a large population including persons such as butchers, veterinarians and those involved in the trade of animal by-products will be exposed to the risk of salmonella infections.

The direct method of infection involves people who eat such uncooked or partially cooked meat which in India is usually from sheep and goats. In this connexion, Khan (1961) stated that in some countries liver of sheep and goats is mixed with bile and eaten raw. In the course of the present study it was observed that the Banjara tribe collected blood from the slaughterhouse for human consumption, a custom which is obviously fraught with danger.

In India, pets such as dogs and cats are often offered raw offal. Similarly, pigs who act as scavengers can pick up the infection from the excreta of infected animals or from their carcasses and may consequently transmit the disease to man, with or without themselves suffering from the infection. It is not unusual to offer the offal or blood of slaughtered animals to poultry in India.

Indirect agents such as flies, cockroaches, fleas and ticks may also aid in the transmission of infections from these sources (Eskey, Prince & Fuller, 1949; Graffer & Mertens, 1950; Gerberich, 1952).

The fact that over 3 % of sheep and goats were found to be infected in this investigation indicates that these carcasses may cause human infection. However, the precise investigation of the relationship between such a potential source and the occurrence of human disease requires that salmonellas should be 'finger-printed' by phage typing.

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Elevated temperature technique for the isolation of salmonellas from sewage and human faeces

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SUMMARY

Modified Moore's swabs, placed in sewers for 5 days, were used to concentrate salmonellas from sewage. Duplicate cultures of swab strips in selenite broth were incubated at 41 and 37° C. respectively. *Salmonella* organisms were recovered consistently from the swabs when the enrichment broths were incubated at 41° C. However, when equal portions of the same swabs were incubated at 37° C., only 22 % of them yielded *Salmonella* organisms. These results indicate an advantage in incubating the selenite broths at 41° C. rather than 37° C. in attempting to isolate salmonellas from sewage.

One hundred and fifty faecal samples were examined for salmonellas by culture in selenite broths incubated at 41 and 37° C. Twelve (8 %) samples were positive at 41° C. compared to only 10 (6·7 %) positive samples at 37° C. This difference is not statistically significant to indicate an advantage of the elevated-temperature of incubation over the conventional temperature in attempting to isolate salmonellas from human faeces. Moreover, results of the recovery rates of *S. paratyphi* B, *S. typhi*, and *S. typhimurium* indicate that an incubation temperature of 37° C. is more appropriate for recovering salmonellas from artificially infected faecal samples than an incubation temperature of 41° C. This stresses the inability of laboratory studies to mimic conditions in nature.

INTRODUCTION

The need for rapid isolation of salmonellas from sewage, faeces and suspect foods has led to the introduction of several selective media. Various workers in this field have claimed the superiority of one or the other medium. Although elevated-temperature techniques have been mainly used to isolate thermophilic organisms (Wilson & Miles, 1964), a study of the literature will show that some authors have increased the selectivity of their media for mesophilic bacteria by raising the incubation temperature of their enrichment broths (Morris & Dunn, 1970; Wilson, 1938).

Harvey & Thomson (1953) increased the isolation rate of *Salmonella* from faeces cultured in selenite-enrichment broth by raising the incubation temperature. Spino (1966), using modified Moore's swabs, reported that *Salmonella* organisms were recovered consistently from surface waters of streams when the enrichment

broths were incubated at 41·5° C. Likewise, Morahan & Hawkesworth (1969) used modified Moore's swab technique to concentrate salmonellas from streams. The subsequent enrichment of gauze strips in enrichment broths at 41° C. yielded 10 *Salmonella* serotypes.

This present study was undertaken to evaluate the comparative efficiency of the elevated, 41° C., and the conventional, 37° C., temperatures of incubation of selenite-enrichment cultures on the isolation of salmonellas from sewage and human faeces. It is a part of a series of investigations into the occurrence and incidence of *Salmonella* serotypes in Lebanon. Isolations from humans and from animals have been previously reported (Nabbut & Jamal, 1970).

MATERIALS AND METHODS

Cultural methods

Modified Moore's swabs (Moore, 1948; Spino, 1966), having remained in sewers along the coast of the city of Beirut for 5 days, were removed from the sampling points and were placed in sterile beakers. They were collected at weekly intervals, brought to the laboratory and processed within 1 hr. of their collection. Strips, containing several layers of gauze cloth, were cut from the swab with flamed scissors, and added to flasks containing 300 ml. of selenite-enrichment broth. Approximately one-half of each swab was used to inoculate one enrichment broth and the other half to inoculate another enrichment broth. The two broth cultures were incubated for 24 hr. at 37 and 41° C. respectively. After incubation two SS plates were streaked from each enrichment culture and incubated at 37° C. for 24 hr. Non-lactose-fermenting colonies thought to be salmonellas were further identified by means of biochemical and serological tests according to standard methods (Edwards & Ewing, 1962).

Specimens of faeces submitted to the bacteriology section of the American University Hospital Laboratories, (AUHL) for bacteriological culture, were examined for the presence of salmonellas. Approximately 2 g. of each faecal specimen was added to 20 ml. of selenite broth in a universal bottle to give a 10 % suspension. This suspension was then shaken manually and 10 ml. volumes were then added to a pair of universal bottles one of which was incubated for 24 hr. at 37° C. whereas the other was incubated at 41° C. After incubation, 2 SS plates were streaked from each enrichment culture and incubated at 37° C. for 24 hr. Colonies resembling *Salmonella* were further identified according to standard procedures (Edwards & Ewing, 1962).

Recovery of salmonellas from artificially infected faecal specimens

A group of 50 faecal specimens, obtained from the parasitology section of the AUHL, were screened to ascertain the absence of salmonellas. Each specimen was diluted with selenite broth to give a 10 % suspension which was then distributed, in 10 ml. volumes, into six universal bottles. Appropriate dilutions of a 24 hr. nutrient broth culture of *Salmonella typhimurium*, *S. typhi* and *S. paratyphi* B that contained about 20 organisms per ml. were separately added in 1 ml. volumes to two bottles of the faecal suspension respectively. Three of the artificially infected

10 % faecal suspensions each containing one of the *Salmonella* species were incubated at 37° C. for 24 hr. whereas the other three bottles were incubated at 41° C. for 24 hr. The enrichment cultures were then streaked on SS plates with a 4 mm. platinum loop. After incubating the plates at 37° C. for 24 hr., colonies resembling salmonellas were tested by slide-agglutination with anti-O-serum of the particular species of *Salmonella* with which the enrichment broth faecal suspension had been inoculated.

Comparative growth of enteric bacteria at 37° C. and 41° C.

The amounts of growth of *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *S. paratyphi* B, *S. typhi* and *S. typhimurium* were compared at 37° C. and 41° C. in selenite broth. Each of the six organisms was grown in nutrient broth for 24 hr. at 37° C. and 0·1 ml. volumes of the appropriate dilutions of each organism were added to duplicate 10 ml. selenite broth in universal bottles. One of the bottles was incubated at 37° C. and the other at 41° C. for 24 hr. Each culture was then diluted serially to 10^{-11} in tubes containing 9 ml. of nutrient broth. All dilutions were incubated at 37° C. for 24 hr. Presence or absence of growth was determined by observing tubes for visible turbidity.

RESULTS

Sixteen *Salmonella* serotypes were isolated from all swab enrichment cultures incubated at 41° C. whereas only four (22 %) of the corresponding swab cultures incubated at 37° C. yielded *Salmonella* organisms (Table 1). Incubation at 41° C. often resulted in an almost pure culture of salmonellas and in a marked reduction in the growth of coliform, proteus and pseudomonas organisms compared with incubation at 37° C.

Table 2 summarizes the comparative results from 12 (8 %) out of 150 faecal cultures that were salmonella positive. Twelve (8 %) *Salmonella* isolates were recovered when the faecal enrichment cultures were incubated at 41° C. compared to 10 (6·7 %) isolates recovered from the corresponding faecal cultures incubated at 37° C.

The recovery rates of *S. paratyphi* B, *S. typhi* and *S. typhimurium* from 50 artificially infected faecal samples is shown in Table 3. It is observed that the total number of recoveries of the three *Salmonella* serotypes was always higher when the faecal enrichment cultures were incubated at 37° C. than when corresponding cultures were incubated at 41° C.

The effects of the incubation temperature on the growth of *S. paratyphi* B, *S. typhi*, *S. typhimurium*, *Proteus mirabilis*, *P. aeruginosa* and *Esch. coli* in selenite broths were determined. It is apparent from Table 4 that incubation at 41° C. is slightly inhibitory for all six organisms and more so for *Proteus mirabilis*, *P. aeruginosa* and *S. typhi*.

Table 1. *Effect of incubation temperature on the isolation of salmonellas from sewage using selenite enrichment broth*

	Salmonella serotypes isolated		Incubation temperature	
Group	Serotype	Number of isolations	37° C.	41° C.
B	*S. eppendorf*	1	+	+
B	*S. essen*	1	+	+
B	*S. paratyphi* B	1	+	+
B	*S. paratyphi* B	1	−	+
C1	*S. livingstone*	1	−	+
C1	*S. montevideo*	2	−	+
C2	*S. bovismorbificans*	1	−	+
C3	*S. sunnycove*	1	+	+
D1	*S. goeteborg*	2	−	+
E1	*S. amsterdam*	1	−	+
E1	*S. butantan*	1	−	+
E1	*S. fuhlsbuettel*	1	−	+
E1	*S. muenster*	1	−	+
E1	*S. nchanga*	1	−	+
E1	*S. sekondi*	1	−	+
M	*S. croft*	1	−	+
	Total	18	4[a]	18[a]

− = Salmonella not isolated.
+ = Salmonella isolated.
a = The difference between 18 and 4 is statistically significant.

Table 2. *Effect of incubation temperature on the isolation of salmonellas from 150 faeces using selenite enrichment broth*

	Salmonella serotypes isolated		Incubation temperature	
Group	Serotype	Number of isolations	37° C.	41° C.
B	*S. sandiego*	1	+	+
B	*S. typhimurium*	1	−	+
B	*S. typhimurium*	1	+	+
C1	*S. tennessee*	2	+	+
C2	*S. manhattan*	1	+	+
C3	*S. kentucky*	1	+	+
D1	*S. goeteborg*	2	+	+
E1	*S. anatum*	2	+	+
E1	*S. anatum*	1	−	+
	Total	12	10[a]	12[a]

+ = Salmonella isolated.
− = Salmonella not isolated
a = The difference between 12 and 10 is not statistically significant.

Table 3. *Effect of incubation temperature on total number of recoveries of* Salmonella paratyphi *B,* S. typhi *and* S. typhimurium *added* to* 10 % *faecal suspensions in selenite broth*

Salmonella serotype	Number of samples examined	Total number positive when incubated at					
		37° C. and 41° C.		37° C.		41° C.	
		Number	%	Number	%	Number	%
S. paratyphi B	50	32	64	29	58	17	34
S. typhi	50	20	40	20	40	5	10
S. typhimurium	50	35	70	34	68	25	50

* Approximately 20 organisms were added to 10 ml. of the faecal suspensions.

Table 4. *Effect of incubation temperature on the growth of* Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella paratyphi *B,* S. typhi *and* S. typhimurium *in selenite broth*

Organism	No./ml. of selenite broth	Incubation temperature	
		37° C.	41° C.
Escherichia coli	220	10^{-9}*	10^{-8}
	220	10^{-9}	10^{-8}
Proteus mirabilis	220	10^{-9}	10^{-5}
	200	10^{-9}	10^{-6}
Pseudomonas aeruginosa	900	10^{-9}	10^{-4}
	100	10^{-8}	10^{-4}
Salmonella paratyphi B	50	10^{-8}	10^{-9}
	100	10^{-9}	10^{-9}
S. typhi	260	10^{-8}	10^{-4}
S. typhi (different strain)	200	10^{-9}	10^{-7}
S. typhimurium	750	10^{-9}	10^{-8}
	200	10^{-9}	10^{-8}

* Values indicate the highest dilution to yield growth when subcultured to nutrient broth.

DISCUSSION

A comparison was made of the effects of different incubation temperatures on the isolation of salmonellas from sewage and human faeces. The enrichment of swab cultures at 41° C. was found to be appropriate for the isolation of salmonellas from sewage. This is in agreement with reports by other workers (Spino, 1966; Harvey & Price, 1968; Morahan & Hawksworth, 1969).

The elevated-temperature of incubation seems to suppress the competing Gram-negative bacteria and to permit *Salmonella* organisms to grow in a relatively pure culture, thus providing an advantage for recognizing the salmonellas. *Esch. coli, Pseudomonas aeruginosa, Proteus, Klebsiella, Aerobacter* and other enteric bacteria are normally found in sewage and are troublesome organisms in attempts to isolate salmonellas from sewage. The incubation of selenite-enrichment broth at 41° C. was found to be slightly inhibitory to *Esch. coli, Salmonella typhimurium,* and

more so for *Proteus mirabilis*, *P. aeruginosa* and *S. typhi* (Table 4). The elevated-temperature technique is, therefore, a factor contributing to the selectivity of the growth of *S. paratyphi* B and *S. typhimurium*, but not for the growth of *S. typhi*. It is recommended for the isolation of salmonellas from sewage and river water, because they contain relatively few salmonellas and a large number of Gram-negative competing organisms of faecal origin.

The elevated-temperature technique may have practical application in epidemiological studies by providing a greater yield of salmonellas from natural waters and sewage. The occurrence of salmonellas in various sewage outfalls and surface waters is important as evidence of an existing health hazard in the population from which the sewage is derived and is directly related to the degree of endemicity of salmonellosis found in a certain community.

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H phase change of *Salmonella thompson* at different temperatures

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SUMMARY

H phase II → phase I conversion of *Salmonella thompson* was studied at different temperatures. All strains were isolated from one outbreak, and a convenient method of phase conversion was sought. In our hands this was best effected at 20° C.

INTRODUCTION

In 1959, an outbreak of salmonella food poisoning occurred in a junior school following a Christmas party. The suspected vehicle of infection was trifle prepared from eggs. Some victims of the incident harboured two serotypes: *S. typhimurium* and *S. thompson* (Harvey, Price, Davis & Morley-Davies, 1961).

All strains isolated were examined for both phase I and phase II H antigens, in case other serotypes might be discovered. We have found this necessary from past experience. No difficulty was encountered in identifying strains of *S. typhimurium*. All cultures of *S. thompson* were in H phase II and when phase change was attempted at 37° C., this was unsuccessful and very prolonged. The method of effecting H phase II → phase I transformation was by the soft agar filled capillary pipette described by Harvey & Price (1961). One such pipette was left, by accident, at room temperature after inoculation. The desired H antigen change of *S. thompson* (1,5 → k) took place within a few days. This fortuitous result encouraged us to study the effect of different temperatures on all phase transformations conducted on strains of *S. thompson* isolated in the outbreak.

Incidents caused by this serotype were not particularly common in our area in 1959 and since regulations for pasteurization of egg products came into being in 1964 they are very seldom seen. We are, therefore, presenting a brief record of a technical facet of an incident occurring many years ago for it seems improbable that we shall have an opportunity of studying this phenomenon again.

MATERIALS

Single colonies were picked from brilliant green MacConkey agars and selected as presumptive *S. thompson* strains by O antigen identification. Purification was necessary as single colonies were sometimes mixtures of *S. typhimurium* and *S. thompson*. Mixed colonies are often found in samples likely to contain multiple serotypes. On one occasion a single colony from a selective agar seeded with

tortoise faeces proved to be a mixture of four different serotypes. When purified, *S. thompson* strains were inoculated to moist agar slopes. These provided the material for study.

METHODS

The technique of H antigen phase change was modified from that described previously (Harvey & Price, 1961). Bijoux bottles containing 0·1 % nutrient agar in 4 ml., amounts were prepared. Davis New Zealand agar was used. The concentration employed was less than that usually recommended for orthodox Craigie tubes. Four drops (4 × 0·02 ml.) of Standards Laboratory polyvalent H phase II serum were added to one or more of these bijoux bottles depending on the number of phase changes to be made. Agar and serum were carefully mixed to avoid bubble formation by rotation of the bottles. A sterile unplugged capillary pipette was then filled with serum-agar mixture to the level of the junction of stem and barrel. After filling, a small amount of dense suspension of culture to be examined was sucked into the terminal 1 cm. of stem. This culture suspension had to be in intimate contact with the mixed agar and serum above it. By manipulation of a teat on the pipette barrel, an air bubble was sucked up under the culture suspension and the stem end was sealed off in a bunsen flame. The teat was removed and the pipette placed in a test tube with cotton wool on the bottom. After all pipettes had been filled, the barrel ends were sealed off in a bunsen flame. Sealing was essential to prevent evaporation as many phase changes were prolonged. It was most simply effected by melting the extreme end of the pipette barrel in a flame, attaching another short piece of glass tubing, pulling out the molten section to a capillary, cooling, cutting and sealing in a bunsen as before.

Two series were studied. In the first, the passage of *S. thompson* through the agar columns was observed at 27 and 37° C. The samples were paired. In the second series the two temperatures investigated were 20 and 27° C. As soon as a strain had reached the upper surface of the agar column, the pipette barrel was opened by scoring with a carborundum cutter and touching the score with a red hot glass rod. The fracture of the barrel occurred at the score. Subculture from the agar surface was made to a small agar slope. This was incubated and the H phase of *S. thompson* identified.

RESULTS

The results are presented in Tables 1 and 2. Two effects of temperature were observed – success or failure to change phase and relative speed of travel of the culture through the column of agar. In the first table, out of 56 paired tests conducted at 27 and 37° C., 38 changed phase at 27° C. only, 8 changed at both temperatures and 0 at 37° C. only. The corresponding figures out of a total of 150 tests at 20 and 27° C. were 19, 130 and 1. Table 2 records the association of temperature with relative speed of travel of the cultures. In the first series, 21 cultures passed more rapidly through the agar at 27° C. than at 37° C., and 31 strains migrated more rapidly at 37 than 27° C. Of the 21 strains, all were in phase I, while only 4 out of the 31 cultures had successfully changed phase. In the 20° C./27° C.

Table 1. Salmonella thompson. *Effect of temperature on H phase II–I change*

Temperature	Successful conversion phase II → I
27° C. only	38
37° C. only	0
27 and 37° C.	8
Total tests performed	56
20° C. only	19
27° C. only	1
20 and 27° C.	130
Total tests performed	150

Table 2. Salmonella thompson. *Effect of temperature on rapidity of passage through agar column*

Passage through agar column first at:				Simultaneous appearance at 27 and 37° C.	
27° C.		37° C.			
Phase II → I	No change	Phase II → I	No change	Phase II → I	No change
21	0	4	27	2	2

Passage through agar column first at:				Simultaneous appearance at 20 and 27° C.	
20° C.		27° C.			
Phase II → I	No change	Phase II → I	No change	Phase II → I	No change
66	0	62	4	18	0

series, 66 appeared first at the top of the agar column at 20° C. and the same number at 27° C. Sixty-six incubated at 20° C. were in phase I and 62 incubated at 27° C. were in phase I. Details of cultures appearing simultaneously at 27° C./ 37° C. and 20° C./27° C. are given in the right-hand half of Table 2.

DISCUSSION

This paper merely seeks to present a brief record of a phenomenon encountered many years ago. It is now perhaps of academic interest only as outbreaks caused by *S. thompson* are rare in the United Kingdom.

Stocker (1949), in a limited experiment on a strain of *S. typhimurium*, found that alteration of medium and of temperature of incubation caused no detectable alteration of rate of mutation per bacterial generation. Our findings with a strain of *S. thompson* appear to contrast with this.

S. thompson, in the past, has always given difficulty in our hands in the phase II → I conversion and all cultures encountered have been in the non-specific phase. It is possible, where strains of *S. thompson* are found, that 20° C. might be a convenient temperature to effect phase conversion. We might be criticized on the grounds that we only studied a single strain of *S. thompson* in this investigation.

This is true, but a strain of *S. thompson* isolated from a clinical case of gastro-enteritis in 1972 behaved in exactly the same way. This recent culture changed phase (II → I) at 20° C., but remained in phase II at 37° C. The migration through the pipette was also more rapid at the low temperature than at the high temperature.

We have no information on the behaviour of other serotypes as far as phase change is concerned.

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The geographical distribution of
Salmonella typhi and *Salmonella paratyphi* A and B phage types during the period 1 January 1966 to 31 December 1969

A Report of the

INTERNATIONAL COMMITTEE FOR ENTERIC
PHAGE-TYPING (ICEPT)

(Received 26 June 1972)

CONTENTS

This report has been compiled by the Secretary, Dr P. Nicolle, from the material provided by the Directors of National Centres and of some Regional Centres of the ICEPT. It was delivered at the meeting of the ICEPT during the 10th International Congress of Microbiology on 8 August 1970 in Mexico. The English version was prepared and edited by the Chairman, Dr E. S. Anderson.*

The results are given in alphabetical order for each of the five continents, and for the countries within the continents.

EXPLANATION OF TABLES

For the purpose of comparison, the presentation of the type distribution proposed by the Secretary for previous reports has been adopted. The percentages of phage types in each country are presented in order of frequency, and the resulting lists comprise one, two or three parts as necessary. The first part (*a*) includes the most numerous phage types, that is, those whose total percentage reaches about 90. In the second part (*b*) are the less common types whose individual percentages are still over 0·5. Finally, when the total number of strains is high enough, the third part (*c*) indicates phage types which are very rare in the area concerned, and whose individual percentages are less than 0·5. This method of presentation has the advantage of indicating the distribution of phage types according to their numerical importance.

Type designations

Recognized Vi-phage types have either lettered or numerical designations. For example, E1; F1; 40.

I + IV indicates cultures resistant to all the specific typing adaptations of Vi-phage II of Craigie & Yen (1938*a*, *b*) but sensitive to phages I and/or IV of Craigie & Yen.

Degraded Vi-strains. These cross-react widely with the Vi-typing phages, but do not conform to a specific typing pattern.

Vi-negative. Cultures devoid of Vi antigen, which cannot be typed with Vi-phages.

The name of each Director is given under the individual centres.

All figures in parentheses are percentages.

ALGERIA
Dᴿ F. Pᴀᴘᴀ – Algiers
(1) *Salmonella typhi*

Type distribution by cases: 387

(*a*) A (39·6); E1a (14·25); B2 (12·9); degraded Vi-strains (9·84); 34 (7·86); C1 (3·8); 42 (2·84); Vi-negative (2·84); 38 (2·07); D1 (1·55); I + IV (1·03); F1 (0·78); 46 (0·52); C4 (0·26).

(2) *Salmonella paratyphi A*

Type distribution by cases: 14.
1: 13 strains. 4: 1 strain.

(3) *Salmonella paratyphi B*

Type distribution by cases: 27.
B.A.O.R.: 7 strains. Dundee: 7 strains. Beccles: 5 strains. Taunton: 4 strains. Untypable: 3 strains. 3a: 1 strain.

SOUTH AFRICA (Republic of)

MISS C. G. CROCKER – Pretoria

(1) *Salmonella typhi*

Type distribution by cases: 8585.

(*a*) A (71·17); I + IV (10·84); E1 (6·20).

(*b*) Degraded Vi-strains (5·35); Vi-negative (3·24); D1 (1·14).

(*c*) 40 (0·33); F1 (0·32); 28 (0·30); T (0·18); 29 (0·17); B1 (0·16); D4 (0·14); D7 (0·10); O (0·09); L2 (0·06); G1 (0·02); 46 (0·02); C1 (0·01); C8 (0·01); D6 (0·01); D8 (0·01); E2 (0·01); 45 (0·01).

Type distribution by foci: 5747

(*a*) A (72·64); I + IV (10·59); Degraded Vi-strains (6·34).

(*b*) E1 (4·06); Vi-negative (3·67); D1 (0·82).

(*c*) 28 (0·45); 40 (0·44); F1 (0·29); D4 (0·12); B1 (0·11); D7 (0·1); 46 (0·04); G1 (0·03); L2 (0·03); O (0·03); 29 (0·03); C1 (0·02); C8 (0·02); D6 (0·02); D8 (0·02); T (0·02); 45 (0·02); E2 (0·01).

BRAZIL

DR GOBERT A. COSTA – Rio-de-Janeiro

(1) *Salmonella typhi*

Number of strains examined: 181.

Two main foci and 9 isolated cases.

Type distribution:

(*a*) A (39·22); E1 (30·93); Vi-positive non-sensitive strains (I + IV group) (7·73); T (7·17); F1 (5·50).

(*b*) Degraded Vi-strains (4·97); G (2·20); D1 (0·55); F2 (0·55); 28 (0·55); 38 (0·55).

CANADA

DR W. C. HARRIS – Ottawa

(1) *Salmonella typhi*

Type distribution by cases: 199.

(*a*) E1 (22·5); degraded Vi-strains (10·5); A (9·5); I + VI (8·0); F1 (7·5); C1 (6·5); D1 (5·0); D9 (4·0); 46 (3·5); N + D1 (3·5); M1 (3·0); E4 (2·5); N (2·0); O (2·0).

(*b*) B1 (1·5); C4 (1·5); Vi-negative (1·5); B2 (1·0); 35 (1·0); C2 (0·5); D2 (0·5); E7 (0·5); F4 (0·5); L1 (0·5); 28 (0·5); 45 (0·5).

Type distribution by foci: 166.

(*a*) E1 (22·3); degraded Vi-strains (10·8); A (9·0); I + IV (9·0); C1 (5·4); D1 (5·4); F1 (5·4); N + D1 (4·2); D9 (3·6); M1 (3·6); 46 (3·6); E4 (2·4); B1 (1·8); O (1·8); Vi-negative (1·8).

(*b*) B2 (1·2); C4 (1·2); N (1·2); 35 (1·2); C2 (0·6); D2 (0·6); E7 (0·6); F4 (0·6); L1 (0·6); 28 (0·6); 45 (0·6).

(2) *Salmonella paratyphi B*

Type distribution by cases: 302.

(*a*) Untypable (26·5); 3a1 var. 1 (14·6); 3a (12·2); Worksop (10·6); Battersea (7·3); Beccles (4·3); 1 (4·0); 3a1 (4·0); Taunton (3·3); 1 var. 3 (3·0); 3a var. 4 (3·0).

(b) 3b (2·6); 1 var. 2 (1·0); 3b var. 2 (0·7); 3b var. 3 (0·7); atypical (0·7).

(c) B.A.O.R. (0·3); Dundee (0·3); Dundee var. 1 (0·3); 50 (0·3).

Type distribution by foci: 197.

(a) Untypable (29·4); Worksop (11·7); 3a (11·2); Battersea (9·1); 3a1 var. 1 (7·1); 1 (6·1); 3a1 (4·6); Beccles 4·1; Taunton (3·5); 3a var. 4 (3·0); 3b (3·0).

(b) 1 var. 3 (2·0); atypical (1·0); 1 var. 2 (0·5); 3b var. 2 (0·5); 3b var. 3 (0·5); B.A.O.R. (0·5); Dundee (0·5); Dundee var. 1 (0·5); 50 (0·5).

Dr S. S. Kasatiya – Montreal

(1) *Salmonella typhi*

Type distribution by cases: 159.

(a) Degraded Vi-strains (16·2); E1 (14·3); A (12·8); I+IV (12·0); F1 (10·3); Vi-negative (8·6); C1 (8·0); E4 (5·8); D1 (3·6).

(b) B2 (3·2); B1 (2·7); F2 (1·0); F3 (0·6); 46 (0·6).

(2) *Salmonella paratyphi B*

Type distribution by cases: 37.

(a) 3a (50·0); Worksop (19·0); 3a1 (13·0); Taunton (13·0).

(b) 3b (3·7).

UNITED STATES OF AMERICA

Miss V. Wilson – Atlanta

(1) *Salmonella typhi*

Type distribution by foci: 626.

(a) E1 (23·80); degraded Vi-strains (18·37); C1 (12·46); A (6·54); F1 (4·63); D1 (4·47); I+IV (4·31); Vi-negative (3·67); 46 (3·03); B1 (2·55); D7 (1·75); B2 (1·60); J1 (1·27); N (1·11); M1 (0·95).

(b) D9 (0·79); 38 (0·79); C4 (0·63); E9 (0·63); 35 (0·63).

(c) B3 (0·47); C9 (0·47); D6 (0·47); D8 (0·47); F4 (0·47); T (0·47); C3 (0·31); E2 (0·31); 26 (0·31); 28 (0·31); 40 (0·31); 50 (0·31); D2 (0·15); D Group (0·15); E3 (0·15); G1 (0·15); 25 (0·15); 27 (0·15); 49 (0·15).

WEST INDIES – JAMAICA

Dr L. S. Grant – Mona-Kingston

(1) *Salmonella typhi*

Type distribution by cases: 107.

(a) E1 (54·62); 45 (19·50); Vi-negative (9·90); A (7·37).

(b) C4 (5·52); degraded Vi-strains (2·40).

Type distribution by foci: 86.

(a) E1 (52·00); 45 (20·0); Vi-negative (12·80); C4 (6·20)

(b) A (4·70); degraded Vi-strains (4·0).

INDIA

Dr A. K. Thomas – Kasauli

(1) *Salmonella typhi*

Type distribution by cases: 330.

(a) A (64·50); E1 (21·12); E4 (5·80).

(b) I + IV (2·77); degraded Vi-strains (2·62); K1 (1·50); O (0·72); B2 (0·62).

(c) E9 (0·32).

(2) *Salmonella paratyphi A*

Type distribution by cases: 43

(a) 1 (38·05); 2 (35·82); 3 (14·32).

(b) 6 (10·87); untypable (0·92).

Dr S. L. Bhatia – New Delhi

(1) *Salmonella typhi*

Type distribution by cases: 3,944.

(a) A (67·69); E1 (15·16); O (9·12); K (2·76); D6 (2·63); J (0·65); Vi-negative (0·60); D1 (0·50); M1 (0·43); 28 (0·23); F1 (0·10); G1 (0·02); T (0·02).

(2) *Salmonella paratyphi A*

Type distribution by cases.

(a) 2 (50·34); 1 (42·12).

(b) 6 (6·16); untypable (1·02).

(c) 5 (0·34).

JAPAN

Dr H. Fukumi – Tokyo

(1) *Salmonella typhi*

Type distribution by cases: 1284.

(a) D2 (25·3); degraded Vi-strains (12·1); H (11·4); E1 (10·4); M1 (10·3); D1 (6·7); E4 (4·6); Vi-negative (3·8); B2 (2·5); E11 (2·4); 39 (2·4).

(b) I + IV (2·0); D6 (1·9); A (1·2); 46 (0·8).

(c) C5 (0·4); B1 (0·2); B8 (0·2); E7 (0·1); L1 (0·1); M3 (0·1); N (0·1); O (0·1); 25 (0·1); 41 (0·1).

Type distribution by foci: 853.

(a) D2 (26·5); degraded Vi-strains (14·4); M1 (12·0); E1 (10·7); D1 (6·7); Vi-negative (6·7); E4 (4·2); I + IV (3·6); B2 (2·9); H (2·8).

(b) D6 (2·5); A (1·9); E11 (1·3); 39 (1·8); 46 (1·2); C5 (0·5).

(c) B1 (0·4); D8 (0·2); E7 (0·1); L1 (0·1); M3 (0·1); N (0·1); O (0·1); 25 (0·1); 41 (0·1).

(2) *Salmonella paratyphi B*

Type distribution by cases: 145.

(a) 3a (43·4); 1 (24·8); untypable (15·9); 3a1 (4·1).

(b) Beccles (2·8); Taunton (2·8); Dundee (2·8); 3b (1·4); B.A.O.R. (1·4); 2 (0·7).

Type distribution by foci: 141.

(*a*) 3a (44·0); 1 (24·8); untypable (15·6); 3a1 (4·3).

(*b*) Beccles (2·8); Dundee (2·8); Taunton (2·1); 3b (1·4); B.A.O.R. (1·4); 2 (0·7).

(3) *Salmonella paratyphi A*

Type distribution by cases: 83.

(*a*) 4 (68·7); untypable (20·5).

(*b*) 1 (8·4); 2 (1·2); 3 (1·2).

Type distribution by foci: 79.

(*a*) 4 (67·1); untypable (22·75).

(*b*) 1 (8·9); 2 (1·3); 3 (1·3).

AUSTRALIA

MISS J. TAPLIN – Melbourne

(1) *Salmonella typhi*

Type distribution by cases: 175.

(*a*) E1 (33·7); D1 (10·7); A (9·4); I+IV (9·0); F1 (8·7); C1 (8·3); degraded Vi-strains (7·7); C4 (2·5).

(*b*) J1 (1·7); M1 (1·4); 40 (1·0); N (0·9); C2 (0·7); D6 (0·7); E9 (0·7); 38 (0·7); 46 (0·7); O (0·6); D4 (0·51).

(*c*) 35 (0·3).

Type distribution by foci: 104.

(*a*) E1 (31·4); A (13·6); degraded Vi-strains (10·9); D1 (8·4); I+IV (7·5); C1 (6·9); F1 (5·2); C4 (1·8); D4 (1·7); M1 (1·7).

(*b*) O (1·7); 40 (1·7); J1 (1·6); D6 (0·9); E9 (0·9); 46 (0·9); C2 (0·8); N (0·8); 35 (0·8); 38 (0·8).

(2) *Salmonella paratyphi B*

Type distribution by human cultures: four cultures, all type Taunton.

Type distribution in water samples or sewage.

Taunton: 22 strains. 1: 8 strains. 1 var. 6: 1 strain. 1 var. 1: 1 strain. 3a: 1 strain. 3a1 var. 1: 1 strain.

POLYNESIA

TONGA (Friendly Islands)

153 strains isolated from 1967 to 1969.

E1 (83·1); A (14·3); I+IV (1·9); degraded Vi-strains (0·7).

AUSTRIA

DR W. ROSCHKA – Graz

(1) *Salmonella typhi*

Type distribution by cases: 226.

(*a*) A (27·43); D (22·56); E (17·69); Group I+IV (10·61); F (8·84); degraded Vi-strains (4·42).

(*b*) B (3·09); C (3·09); N (1·32).

(*c*) T (0·44); 28 (0·44).

(2) *Salmonella paratyphi B*

Number of strains examined from all origins: 1509.

Type distribution:

(*a*) Taunton (36·24); Dundee (36·18); 1 (9·21); B.A.O.R. (4·17); degraded strains (2·91); 3a (2·31).

(*b*) 3b var. 3 (1·78); 3a var. 2 (1·52); 3a1 (1·45); 3b var. 2 (0·86); Beccles (0·86); 3a1 var. 1 (0·59); 3a1 var. 2 (0·53); 3b (0·53).

(*c*) Jersey (0·33); 1 var. 1 (0·19); 1 var. 2 (0·19); Worksop (0·06).

BELGIUM
PROF. J. BEUMER – Brussels

(1) *Salmonella typhi*

Type distribution by cases: 83.

(*a*) C1 (31·3); E1 (24·1); A (12·0); D1 (6·0); 46 (6·0); degraded Vi-strains (3·6).

(*b*) C5 (2·4); D4 (2·4); D9 (2·4); 28 (2·4); I+IV (2·4); Vi-negative (2·4); F1 (1·2); 40 (1·2).

Type distribution by foci: 75.

(*a*) C1 (32·0); E1 (20·0); A (13·3); D1 (6·7); 46 (6·7); degraded Vi-strains (4·0).

(*b*) D4 (2·7); D9 (2·7); 28 (2·7); I+IV (2·7); Vi-negative (2·7); C5 (1·3); F1 (1·3); 40 (1·3).

(2) *Salmonella paratyphi B*

Type distribution by cases: 37.

(*a*) 3a1 (32·4); Dundee (29·7); Jersey (21·6); 1 (8·1).

(*b*) Untypable (5·4); 3a (2·7).

Type distribution by foci: 36.

(*a*) 3a1 (30·6); Dundee (30·6); Jersey (22·2); 1 (8·3).

(*b*) Untypable (5·5); 3a (2·8).

BULGARIA
DR ROSA COHEN – Sofia

(1) *Salmonella typhi*

Type distribution by cases: 128.

(*a*) E1 (25·00); A (17·18); I+IV (16·40); F1 (15·62); Vi-negative (15·62).

(*b*) D1 (7·81); degraded Vi-strains (1·56); 40 (0·78).

Type distribution by foci: 114.

(*a*) E1 (21·05); A (19·29); F1 (16·66); I+IV (16·66); Vi-negative (15·78).

(*b*) D1 (7·89); degraded Vi-strains (1·75); 40 (0·87).

CZECHOSLOVAKIA
DR J. BORECKA – Bratislava

(1) *Salmonella typhi*

Type distribution by cases: 2050.

D1 (25·50); E1 (21·42); A (13·94); F1 (8·57); degraded Vi-strains (7·63); C1 (6·75); I+IV (3·56); 46 (2·83); D9 (2·76); Vi-negative (1·82); D6 (1·60); D4 (1·02);

28 (0·87); C4 (0·36); 40 (0·36); D2 (0·30); F5 (0·22); B2 (0·14); 27 (0·14); 32 (0·14); C5 (0·07).

Type distribution by foci: 1007.

E1 (22·64); D1 (21·94); A (13·90); F1 (8·94); degraded Vi-strains (8·84); C1 (5·86); I+IV (4·47); 46 (2·98); Vi-negative (2·48); D9 (1·69); D6 (1·59); D4 (1·29); 28 (1·09); C4 (0·50); 40 (0·49); D2 (0·40); F5 (0·30); B2 (0·20); 27 (0·20); C5 (0·10); 32 (0·10).

(2) *Salmonella paratyphi B*

Type distribution by cases: 503.

Taunton (33·11); Beccles (19·82); untypable (13·51); 1 var. (7·66); 1 (6·75); 3a1 (3·60); 3a (3·15); B.A.O.R. (2·93); Dundee (2·93); 3a1 var. (2·70); 3a var. ? (2·03); 3b (0·90); 3b var. ? (0·43); Beccles var. ? (0·23); Dundee var. ? (0·23).

Type distribution by foci: 374.

Taunton (38·50); untypable (16·04); 1 var. ? (9·09); 1 (8·02); Beccles (5·61); 3a1 (4·28); 3a (3·74); B.A.O.R. (3·48); Dundee (3·48); 3a1 var. ? (3·21); 3 var. ? (2·40); 3b (1·07); 3b var. ? (0·54); Beccles var. ? (0·27); Dundee var. ? (0·27).

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FINLAND

DR P. H. MÄKELA – Helsinki

</div>

(1) *Salmonella typhi*

Type distribution by cases: 34.

(*a*) B2 (38·23); C4 (23·52); D1 (11·76); A (5·88); 50 (5·88); Vi-negative (5·88).

(*b*) E1 (2·94); F1 (2·94); 40 (1·94).

Type distribution by foci: 15

D1 (26·66); A (13·33); 50 (13·33); Vi-negative (13·33); C4 (6·66); D2 (6·66); E1 (6·66); F1 (6·66); 40 (6·66).

(2) *Salmonella paratyphi B*

Type distribution by cases: 407.

(*a*) Taunton (64·5); 3a1 var. 1 (20·2); N.S.T. (7·4).

(*b*) 3a var. 4 (1·7); untypable (1·5); 3a (1·00); 1 (0·7); 3b (0·7); Beccles (0·7).

(*c*) 1 var. 5 (0·2); 3a1 (0·2); Battersea (0·2); Beccles (var. 3) (0·2); Jersey (0·2); Worksop (0·2).

Type distribution by foci: 290.

(*a*) Taunton (60·00); 3a1 var. 1 (22·0); N.S.T. (9·0).

(*b*) 3a var. 4 (2·1); untypable (2·1); Beccles (1·0); 1 (0·7); 3b (0·7).

(*c*) 1 var. 5 (0·3); 3a (0·3); 3a1 (0·3); Battersea (0·3); Beccles var. 3 (0·3); Jersey (0·3); Worksop (0·3).

FRENCH CENTRE

Dr P. Nicolle, from 1966 to 1968 (inclusive)
Dr J.-F. Vieu in 1969

(I) FRANCE

(1) *Salmonella typhi*

Type distribution by cases: 1024.

(a) E1 (25·78); A (16·59); C1 (15·23); degraded Vi-strains (12·20); D1 (6·45); F1 (4·50); B2 (4·20); Vi-negative (2·93); 46 (2·64);

(b) C4 (1·37); D4 (1·27); 42 (1·30); C5 (0·87); L2 (0·59); I + IV (0·59).

(c) Central African variety of phage-type C1 (0·49); T (0·48); 34 (0·39); D2 (0·30); F5 (0·30); G1 (0·30); C3 (0·29); N (0·19); 29 (0·20); C2 (0·09); D6 (0·09); E3 (0·09); F4 (0·09); J1 (0·09); 41 (0·09).

(2) *Salmonella paratyphi B*

Type distribution by cases: 772.

(a) Dundee (35·49); Taunton (19·56); 1 (16·97); Jersey (8·94); 3a1 (5·57); untypable (5·18).

(b) Beccles (4·54); 3b (1·95); 3a (0·76); B.A.O.R. (0·64).

(c) Battersea (0·39).

(3) *Salmonella paratyphi A*

Total numbers of strains: 291.

France: 23 cultures; phage-types 1, 2 and 4.

Algeria: 24 cultures; phage-types 1, 2 and 4.

Morocco: 68 cultures; phage-types 1 and 2.

Senegal: 2 strains; phage-type 1.

Egypt: 115 cultures; phage-types 1 (29·56); 2 (46·95); and 4 (16·52); untypable (6·96).

Iran: 1 strain; phage-type 6.

Syria: 1 strain; phage-type 2.

Israel: 26 strains; phage-types 1, 2 and 4.

Cambodia: 7 strains; phage-type 1 and untypable group.

Vietnam: 1 strain; phage-type 1.

Turkey: 23 strains; phage-types 1 and 2 (the strains of phage-type 2 represent 95·65 % of the cultures considered).

(II) NORTH AFRICA*

Morocco (strains sent to the French Centre for Enteric Phage-typing by the Institut Pasteur of Casablanca and of Tangiers).

(1) *Salmonella typhi*

Type distribution by cases: 485.

(a) C1 (53·61); A (15·88); E1 (9·69); degraded Vi-strains (6·60); D1 (4·75).

* The strains of *S. para A* isolated in these different countries are included in paragraph 3 of section I of the French report.

(b) 42 (2·68); Vi-negative (2·06); B2 (1·86); C5 (0·83); D4 (0·62).

(c) E2 (0·42); L2 (0·42); D2 (0·21); O (0·21); 40 (0·21).

(2) *Salmonella paratyphi B*

Number of cases considered: 9

Type distribution.

Taunton: 5 strains. 3a1: 2 strains. Beccles: 2 strains.

Algeria (see the report of Dr F. Papa of the Institut Pasteur of Algeria).

Tunisia* (strains sent to the French Centre for Enteric Phage-typing, by the Institut Pasteur of Tunis).

Salmonella typhi

Type distribution by cases: 267.

(a) E1 (58·43); A (25·84); 42 (5·99).

(b) degraded Vi-strains (4·50); Vi-negative (3·37); O (1·12).

(c) D6 (0·38); 34 (0·38).

Egypt* (strains sent to the French Centre by Namru-3-Cairo).

(1) *Salmonella typhi*

Type distribution by cases 225.

(a) Degraded Vi-strains (23·56); 40 (17·78); C1 (10·22); D1 (8·44); I + IV (7·11); Vi-negative (6·67); A (6·22); E1 (4·89); 42 (4·0); J1 (2·67).

(b) E2 (2·22); G1 (1·78); T (1·33); C5 (0·88).

(c) F3 (0·44); L2 (0·44); O (0·44); 36 (0·44); 50 (0·44).

(2) *Salmonella paratyphi B*

Type distribution of 5 strains: Taunton (3 strains); Dundee (2 strains).

(III) FRENCH-SPEAKING BLACK AFRICA*

Senegal (strains received from the Institut Pasteur of Dakar).

Salmonella typhi:

Type distribution by cases: 269.

(a) A (46·47); E1 (24·91); Vi-negative (10·41); C4 (6·32); degraded Vi-strains (3·72).

(b) D1 (3·35).

Ivory Coast (strains received from the Institut Pasteur of Abidjan).

Salmonella typhi

Type distribution by cases: 214.

(a) A (42·51); C1 (19·15); degraded Vi-strains (14·02); Vi-negative (11·22); D1 (5·14).

(b) E1 (3·73); 42 (2·80).

(c) C2 (0·46); C4 (0·46); D6 (0·46); 29 (0·46).

*Upper Volta** (strains received from the Institut Pasteur of Ouaga Dougou).

Salmonella typhi

   Type distribution by cases: 89.
   (a) A (76·40); E1 (10·11); Degraded Vi-strains (4·49).
   (b) C1 (3·37); D6 (2·24); Vi-negative (2·24); 42 (1·12).

*Republic of Chad and Central African Republic**

Salmonella typhi

   Type distribution by cases: 38.
   (a) Central African variety of phage-type C1 (78·94); Vi-negative (10·52).
   (b) A (7·89); E1 (2·63).

*Cameroon** (strains received from the Institut Pasteur of Yaounde).

Salmonella typhi

   Type distribution by cases: 133.
   (a) A (79·70); C1 (9·77).
   (b) Vi-negative (5·26); D1 (1·50); C4 (0·75); F1 (0·75); 34 (0·75); 35 (0·75);
degraded Vi-strains (0·75).

Congo Zaïre and Congo Brazzaville (strains received from Dr Van Oye of the
Institut Pasteur of Brazzaville).

Salmonella typhi

Type distribution by cases: 124.
   (a) A (35·48); E1 (31·45); Central African variety of phage-type C1 (12·90);
degraded Vi-strains (11·29).
   (b) O (5·64); Vi-negative (1·61); B2 (1·24); I + IV (1·24).

Madagascar (Malagasy Republic) (strains received from the Institut Pasteur of
Tananarive).

Salmonella typhi

   Type distribution by cases: 18.
   (a) E1 (53·76); A (38·17).
   (b) Vi-negative (3·76); degraded Vi-strains (2·69); C1 (1·61).

<div align="center">(IV) MIDDLE EAST</div>

Turkey (strains received from colleagues in Istanbul & Ankara).

Salmonella typhi

   Type distribution by number of strains: 7.
   T: 3 strains. 28: 2 strains. B2: 1 strain. 46: 1 strain.

Iran (Strains received from the Institut Pasteur of Teheran).

Salmonella typhi

   Type distribution by cases: 49.
   (a) Degraded Vi-strains (44·89); I + IV (18·36); F1 (10·20); A (8·16); 28 (6·12).
   (b) D1 (2·04); D6 (2·04); M1 (2·04); 39 (2·04); 40 (2·04); Vi-negative (2·04).

(V) FAR EAST

South Vietnam and Cambodia (strains sent by the Institut Pasteur of Saigon and the Institute of Biology of Phnom-Penh).

Salmonella typhi

Type distribution by cases: 611.

(*a*) I + IV (40·91); degraded Vi-strains (9·32); E1 (7·36); A (6·54); Vi-negative (6·54); D2 (4·90); 37 (3·76); M1 (3·36); D2 (2·45); D6 (1·96); E10 (1·96); 29 (1·47).

(*b*) D1 (0·98); G1 (0·98); M2 (0·64); M4 (0·64); 28 (0·64).

(*c*) D4 (0·48); E7 (0·48); F1 (0·48); J3 (0·48); 46 (0·32); 49 (0·32); C1 (0·16); C5 (0·16); E2 (0·16); E3 (0·16); E9 (0·16); J1 (0·16); L1 (0·16); M3 (0·16); 25 (0·16); 38 (0·16); 39 (0·16).

(VI) AMERICA: CARRIBEAN REGION

Dutch Guyana; French West Indies (Islands of Martinique and Guadeloupe).

Salmonella typhi

(Ninety-three strains sent by the Institut Pasteur of Cayenne, of Fort-de-France, of Pointe-a-pitre and by colleagues from Dutch Guyana)

Type distribution by cases:

(*a*) A (73·11); E1a (10·75); N (8·60)

(*b*) Degraded Vi-strains (5·37); C1 (2·15).

(VII) AUSTRALASIA

New Caledonia

Salmonella typhi (6 strains received from the Institut Pasteur of Noumea).

A: 4 strains. D2: 2 strains.

French Polynesia: Tahiti

Salmonella typhi (29 strains received from the Institut Pasteur of Papeete).
E1a: 15 strains. A: 13 strains. Vi-negative: 1 strain.

GERMANY (FEDERAL REPUBLIC OF)

Dr I. Bohlck – Kiel

(1) *Salmonella typhi*

(*A*) Number of strains examined, isolated from diseased persons or carriers: 119.
Type distribution by cases:

(*a*) E1a (19·30); F1 (16·03); A (15·20); I + IV group (8·40); degraded Vi-strains (7·50); Vi-negative (7·50); E1b (5·90); D1 (5·00); D2 (3·40); 28 (3·40).

(*b*) 46 (3·40); C1 (1·70); N (1·70); D6 (0·80); T (0·80).

(*B*) Type distribution by foci: 109.

(*a*) E1a (18·30); F1 (17·40); A (13·70); I + IV group (8·30); degraded Vi-strains (8·30); Vi-negative (8·30); D1 (5·50); E1b (5·50); D2 (3·70); 46 (3·70).

(*b*) 28 (2·80); N (1·80); C1 (0·90); D6 (0·90); T (0·90).

(2) *Salmonella paratyphi B*

(*A*) Number of strains examined, isolated from diseased persons or carriers: 119. Type distribution by cases:

(*a*) Taunton–Kampen (42·00); 3a1 var. 2 (12·60); 1 (11·80); untypable (10·1); 3a1 (7·60); 3a1 var. 1 (5·00); 3b (2·50).

(*b*) B.A.O.R. (2·50); 3a (1·70); Beccles (1·70); Dundee (1·70); 3b var. 1 (0·80).

(*B*) Type distribution by foci: 99.

(*a*) Taunton–Kampen (34·40); 1 (13·20); untypable (12·10); 3a1 var. 2 (11·10); 3a1 (9·10); 3a1 var. 1 (6·1); 3b (3·0).

(*b*) B.A.O.R. (3·0); 3a (2·0); Beccles (2·0); Dundee (2·0); 3b var. 1 (1·00).

<div align="center">PROF. DR. H. BRANDIS – Bonn</div>

(1) *Salmonella typhi*

(*A*) Number of strains examined, isolated from diseased persons or carriers: 1092.

Type distribution by cases:

(*a*) E1a (21·79); A (13·92); degraded Vi-strains (11·09); F1 (8·42); C1 (6·68); Vi-negative (6·41); D1 (5·49); E1b (5·31); D2 (3·39); 46 (2·93); untypable II (2·93); untypable I (1·94).

(*b*) 38 (1·37); N (1·10); 28 (1·10); F4 (0·82); F5 (0·82); D9 (0·73); D4 (0·64).

(*c*) C4 (0·46); D6 (0·37); T (0·27); 40 (0·27); rough (0·27); B2 (0·18); J1 (0·18); B1 (0·09); E2 (0·09); E7 (0·09); E10 (0·09); 27 (0·09).

(*B*) Type distribution by foci: 950.

(*a*) E1a (22·42); A (14·63); degraded Vi-strains (11·68); F1 (8·11); Vi-negative (6·42); C1 (6·32); D1 (5·68); E1b (5·16); D2 (3·26); 46 (2·95); untypable II (2·95); untypable I (2·00).

(*b*) 28 (1·37); F4 (0·95); N (0·84); D4 (0·74); D9 (0·74); F5 (0·74); 38 (0·53).

(*c*) C4 (0·42); D6 (0·42); 40 (0·32); Rough (0·32); D2 (0·21); T (0·21); B1 (0·11); E2 (0·11); E7 (0·11); E10 (0·11); J1 (0·11); 27 (0·11).

(2) *Salmonella paratyphi B*

(*A*) Number of strains examined, isolated from diseased persons or carriers: 891. Type distribution by cases:

(*a*) Taunton (28·06); B.A.O.R. (18·41); 1 (12·46); 3a1 (6·40); untypable (6·06); Dundee (5·16); 3a (4·94); 3a1 var. 1 (4·71); 3b (4·38).

(*b*) Beccles (3·70); Rough (2·99); 3a var. 4 (1·01); Jersey (1·01); 3a1 var. 4 (0·56).

(*c*) 1 var. 8 (0·11); 3a var. 2 (0·11).

(*B*) Type distribution by foci: 670.

(*a*) Taunton (30·00); B.A.O.R. (14·18); 1 (13·58); 3a1 (6·87); untypable (6·27); 3a (5·67); 3b (5·08); Dundee (4·63); 3a1 var. 1 (3·73).

(*b*) Rough (3·28); Beccles (3·13); 3a var. 4 (1·34); Jersey (1·19); 3a1 var. 4 (0·75).

(*c*) 1 var. 8 (0·15); 3a var. 2 (0·15).

<div style="text-align:center">Dr V. Lenk – Berlin</div>

(1) *Salmonella typhi*

Type distribution by cases: 249.

(*a*) E1a (19·28); A (15·26); degraded Vi-strains (10·04); F1 (8·03); I + IV (8·03); Vi-negative (7·23); D2 (6·02); D1 (5·22); F5 (5·22); C1 (4·02); E1b (2·81).

(*b*) 46 (1·61); D4 (1·20); 28 (1·20); 40 (1·20); T (0·80).

(*c*) B2 (0·40); C4 (0·40); D9 (0·40); F4 (0·40); J1 (0·40); 50 (0·40); Rough (0·40).

Type distribution by foci: 205.

(*a*) E1a (19·51); A (16·10); degraded Vi-strains (10·73); F1 (9·27); Vi-negative (8·29); I + IV (7·80); D1 (5·85); C1 (4·39); D2 (4·39); E1b (2·44); 46 (1·95).

(*b*) 28 (1·46); 40 (1·46); D4 (0·98); F5 (0·98); T (0·98).

(*c*) B2 (0·49); C4 (0·49); D9 (0·49); F4 (0·49); J1 (0·49); 50 (0·49); Rough (0·49).

(2) *Salmonella paratyphi B*

Type distribution by cases: 214.

(*a*) Taunton (34·64); B.A.O.R. (18·69); 3a1 (9·81); 1 (8·88); 3a1 var. (5·61); 3b (5·61); Rough (4·21); untypable (3·74).

(*b*) 3a (2·80); Jersey (2·34); Dundee (2·34); Beccles (1·40).

(*c*) 1 var. (0·47); 3b var. ? (0·47).

Type distribution by foci: 201.

(*a*) Taunton (32·84); B.A.O.R. (18·41); 3a1 (9·45); 1 (8·96); 3a1 var. (5·97); 3b (5·97); Rough (4·48); untypable (3·98).

(*b*) 3a (2·99); Jersey (2·49); Dundee (1·99); Beccles (1·49); 1 var. ? (0·50); 3b var. ? (0·50).

<div style="text-align:center">Dr V. Lenk and Dr B. Schmidt – Berlin</div>

(1) *Salmonella typhi*

Type distribution by cases: 658.

(*a*) E1a (19·15); I + IV (12·61); degraded Vi-strains (11·85); Vi-negative (10·49); A (10·33); F1 (9·88); D2 (5·78); D1 (4·56); E1b (4·56).

(*b*) C1 (2·89); 40 (2·89); 28 (1·52); 46 (0·76); rough (0·61).

(*c*) F4 (0·46); F5 (0·46); J1 (0·30); Vi-positive phage-negative (0·30); C4 (0·15); M1 (0·15); 32 (0·15); 38 (0·15).

Type distribution by foci: 634.

(*a*) E1a (19·24); I + IV (12·93); degraded Vi-strains (11·67); Vi-negative (10·88); A (10·25); F1 (9·94); D2 (5·84); E1b (4·73); D1 (4·57).

(*b*) C1 (3·0); 40 (2·05); 28 (1·26); 46 (0·79); rough (0·63).

(*c*) F4 (0·47); F5 (0·47); J1 (0·32); Vi-positive phage-negative (0·32); C4 (0·16); M1 (0·16); 32 (0·16); 38 (0·16).

(2) *Salmonella paratyphi B*

Type distribution by cases: 274.

(*a*) Taunton (31·75); 3a1 (14·23); 1 (13·14); B.A.O.R. (10·22); 3a (9·12); rough (8·39); 3a1 var. (5·11).

(*b*) Untypable (2·92); Dundee (1·82); 3b (1·09); Jersey (1·09); Beccles (0·73).

(*c*) 3b var. (0·36).

Type distribution by foci: 256.

(*a*) Taunton (33·59); 3a1 (13·28); 1 (12·89); B.A.O.R. (9·38); 3a (8·59); rough (8·59); 3a1 var. ? (5·08).

(*b*) Untypable (3·13); Dundee (1·95); 3b (1·17); Beccles (0·78).

(*c*) 3b var. ? (0·39).

Dr Polanetzki – Frankfurt-on-Main

(1) *Salmonella typhi*

Type distribution by cases: 1173.

(*a*) E1a (14·64); A (11·66); F5 (9·88); degraded Vi-strains (8·43); D1 (6·56); E1b (6·56); F1 (6·56); C1 (5·71); Vi-negative (5·45); untypable I (5·11); 46 (3·49); untypable II (2·81); D2 (2·56).

(*b*) 28 (2·22); D9 (1·88); 40 (0·85); D6 (0·77); F2 (0·76); J1 (0·59); C4 (0·52); E7 (0·52).

(*c*) D4 (0·43); D8 (0·43); B2 (0·34); E10 (0·34); 38 (0·34); N (0·26); D10 (0·17); F4 (0·08); T (0·08).

Type distribution by foci: 896.

(*a*) E1a (17·63); A (12·80); Degraded Vi-strains (9·82); F1 (7·81); C1 (7·24); D1 (7·13); Vi-negative (6·66); E1b (6·47); untypable I (5·91); 46 (3·34); untypable II (3·34); D2 (3·11); 28 (1·89).

(*b*) D6 (1·00); 40 (1·00); F2 (0·89).

(*c*) E7 (0·44); E10 (0·44); J1 (0·44); B2 (0·33); C4 (0·33); D4 (0·33); N (0·33); 38 (0·33); D8 (0·22); D9 (0·22); F5 (0·22); D10 (0·11); F4 (0·11); T (0·11).

(2) *Salmonella paratyphi B*

Type distribution by cases: 955.

(*a*) Taunton (34·56); 3a1 var. 1–2 (11·62); B.A.O.R. (10·16); 1 (8·27); untypable (6·91); 3a1 (6·59); Dundee (5·76); Beccles (4·71).

(*b*) 3a (4·09); 3b (3·25); 1 var. 2 (2·19); 3a Jersey ? (0·73); Jersey (0·52).

(*c*) 3a var. 4 (0·32); 2 (0·21); 3b var. 2 (0·11).

Type distribution by foci: 777.

(*a*) Taunton (33·97); 3a1 var. 1–2 (11·32); B.A.O.R. (9·40); 1 (8·49); untypable (7·10); 3a1 (7·07); Dundee (5·40); Beccles (4·89).

(*b*) 3a (4·24); 3b (3·86); 1 var. 1 (2·32); 3a Jersey ? (0·77); Jersey (0·64).

(*c*) 3a var. 4 (0·24); 2 (0·13); 3b var. 2 (0·13).

DR F. WURSCHING – Munich

(1) *Salmonella typhi*

Type distribution by cases: 549.

A (19·3); E1a (16·2); atypical (14·0); D1 (10·9); F1 (10·2); untypable (5·8); C1 (4·9); Vi-negative (2·9); B2 (2·7); C2 (1·4); E1b (1·4); F5 (1·1); E10 (0·9); 38 (0·9); D2 (0·7); D4 (0·7); D6 (0·7); 28 (0·7); C4 (0·6); T (0·6); 40 (0·6); F4 (0·4); 34 (0·4); 46 (0·4); C5 (0·2); D9 (0·2); E2 (0·2); 27 (0·2); 35 (0·2); 36 (0·2); 42 (0·2); N (0·2).

Type distribution by foci: 449.

A (19·6); E1a (15·2); atypical (14·9); D1 (10·3); F1 (8·3); untypable (6·7); C1 (5·6); Vi-negative (3·1); B2 (2·7); E1b (1·8); C2 (1·3); F5 (1·1); 38 (1·1); D4 (0·9); 28 (0·9); C4 (0·7); D2 (0·7); D6 (0·7); E10 (0·7); T (0·7); 34 (0·4); 40 (0·4); 46 (0·4); C5 (0·2); D9 (0·2); E2 (0·2); F4 (0·2); 27 (0·2); 35 (0·2); 36 (0·2); 42 (0·2); N (0·2).

(2) *Salmonella paratyphi B*

Type distribution by cases: 405.

Taunton–Kampen (40·1); 1 (10·0); 3a1 var. 2 (9·1); B.A.O.R. (8·6); 3b var. 5 (3·2); rough (3·00); 1 (2·2); 1 var. 1 (2·2); 3a1 Leeuwarden (2·2); B.T.6 (2·2); atypical (2·2); Taunton–Kampen var. 1 (2·00); 3a (1·7); untypable (1·7); Jersey (1·5); Dundee (1·0); 3a var. 2 (1·0); Q3 (1·0); 3a var. 6 (0·8); 3a var. 4 (0·8); Beccles 22 (0·5); Midwoud (0·5); S1–54 (0·5); B.T.3 (0·5); 3b (0·2); 3b var. 2 (0·2); 3a1 Schiedam (0·2); P3 (0·2); Q6 (0·2).

Type distribution by foci: 298.

Taunton–Kampen (35·7); 1 (10·1); B.A.O.R. (8·40); 3a1 var.1–2 (8·1); 3b var. 5 (4·0); rough (3·7); B.T.6 (3·0); 1 var. 1 (2·7); 1 (2·3); 3a (2·3); 3a1 Leeuwarden (2·3); atypical (2·3); untypable (2·3); Taunton–Kampen var. 1 (2·0); Jersey (1·7); Dundee (1·6); 3a var. 2 (1·0); 3a var. 4 (1·0); Beccles 22 (0·7); Midwoud (0·7); B.T.3 (0·7); 3b (0·3); 3b var. 2 (0·3); S1–54 (0·3); Sittard (0·3); 3a1 Schiedam (0·3); P3 (0·3); Q3 (0·3); Q6 (0·3).

GERMANY (DEMOCRATIC REPUBLIC OF)

DR H. RISCHE – Wernigerode/Harz

(1) *Salmonella typhi*

Type distribution by cases: 4087.

E1a (21·3); A (9·5); degraded Vi-strains (9·0); Vi-negative (8·6); I+IV (8·1); F1 (7·8); E1b (7·0); D1 (5·9); C1 (5·7); D2 (3·3); F4 (2·6); 46 (2·1); N (1·8); 38 (1·1); T (0·9); 28 (0·9); 40 (0·9); D4 (0·7); D6 (0·4); D9 (0·4); F5 (0·4); F2 (0·3); F7 (0·2); C5 (0·1); E7 (0·1); B2 (0·08); C4 (0·05); D7 (0·05); J4 (0·05); 29 (0·05); 34 (0·05); 36 (0·05); 50 (0·05); B3 (0·02); J1 (0·02); M1 (0·02); 43 (0·02).

Type distribution by foci: 3227.

E1a (20·7); A (9·5); degraded Vi-strains (9·3); Vi-negative (8·5); I+VI (8·4); F1 (7·9); E1b (7·1); D1 (6·3); C1 (5·8); D2 (3·5); F4 (2·6); 46 (2·0); 38 (1·1); D4 (0·9); T (0·9); 28 (0·9); N (0·8); 40 (0·8); F5 (0·5); D6 (0·4); D9 (0·4); F2 (0·3);

F7 (0·2); E7 (0·1); C4 (0·06); C5 (0·06); D7 (0·06); J4 (0·06); 29 (0·06); 34 (0·06); 36 (0·06); B2 (0·03); B3 (0·03); J1 (0·03); M1 (0·03); 43 (0·03); 50 (0·03).

(2) *Salmonella paratyphi B*

Type distribution by cases: 2269.

Taunton (41·5); 3a1 (15·9); 1 (14·1); B.A.O.R. (7·5); 3b (6·1); untypable (5·7); 3a (5·6); Dundee (2·00); Beccles (0·5); Jersey (0·4).

Type distribution by foci: 1667.

Taunton (41·7); 3a1 (16·9); 1 (14·4); B.A.O.R. (7·3); untypable (6·5); 3a (5·5); 3b (4·2); Dundee (2·1); Beccles (0·6); Jersey (0·4).

<div align="center">HUNGARY</div>

<div align="center">Dr H. Milch – Budapest</div>

(1) *Salmonella typhi*

Type distribution by cases: 6062.

(*a*) E1a (21·20); A (19·91); D1 (19·25); F1 (9·54); degraded Vi-strains (8·60); C1 (6·50); I + IV (3·70); B2 (3·45).

(*b*) Vi-negative (2·65); D2 (0·90); E1b (0·90); 28 (0·82).

(*c*) D4 (0·49); 46 (0·49); C2 (0·32); B1 (0·24); B3 (0·16); D9 (0·16); F5 (0·16); 27 (0·16); C4 (0·08); D6 (0·08); E4 (0·08); 38 (0·08); 43 (0·08).

Type distribution by foci: 714.

(*a*) A (19·60); E1a (18·58); D1 (17·02); F1 (10·08); degraded Vi-strains (9·24); C1 (6·58); B2 (4·48); I + IV (4·20).

(*b*) Vi-negative (3·22); D2 (1·12); E1b (1·12); 28 (0·84); D4 (0·70); 46 (0·70); C2 (0·56).

(*c*) B1 (0·42); B3 (0·28); D9 (0·28); F5 (0·28); 27 (0·28); C4 (0·14); D6 (0·14); E4 (0·14); 38 (0·14); 43 (0·14).

(2) *Salmonella paratyphi B*

Type distribution by cases: 821.

(*a*) Taunton (58·65); 1 (8·84); untypable (6·50); 3a1 var. 2 (5·36); B.A.O.R. (5·36); Dundee (4·59).

(*b*) 1 var. 1 (2·68); 3a1 (2·68); Beccles (1·91); 3a1 var. 1 (1·53); 3a (0·76).

(*c*) 3b (0·38); 3b var. 2 (0·38); 3b var. 3 (0·38).

Type distribution by foci: 161.

(*a*) Taunton (48·44); 1 (11·80); 3a1 var. 2 (8·07); B.A.O.R. (7·48); Dundee (6·85); untypable (6·84).

(*b*) 3a1 (2·48); Beccles (2·48); 1 var. 1 (1·86); 3a (1·24); 3a1 var. 1 (0·62); 3b (0·62); 3b var. 2 (0·62); 3b var. 3 (0·62).

ITALY (Southern)

THE LATE PROFESSOR G. D'ALESSANDRO – Palermo

(1) *Salmonella typhi*

Type distribution by cases: 182.

(a) C1 (40·6); degraded Vi-strains (18·1); A (12·0); C4 (6·5); D1 (6·5); Vi-negative (4·3); C3 (2·7).

(b) E1 (2·1); 46 (1·6); I+IV (1·6); B2 (1·0); E10 (0·5); F5 (0·5); 28 (0·5); 37 (0·5).

Type distribution by foci: 156.

(a) C1 (42·3); A (14·1); degraded Vi-strains (12·1); C4 (7·6); D1 (7·6); C3 (3·2); E1 (2·5).

(b) Vi-negative (2·5); 46 (1·9); I+IV (1·9); B2 (1·2); E10 (0·6); F5 (0·6); 28 (0·6); 37 (0·6).

(2) *Salmonella paratyphi B*

Type distribution by cases: 66.

(a) Untypable (63·6); atypical (9·00); 3a1 var. 3 (7·5); B.A.O.R. (6·0); Dundee (6·0).

(b) 3a1 var. 1 (3·00); 3a1 (1·5); Taunton (1·5).

Type distribution by foci: 46.

(a) Untypable (54·3); atypical (13·0); 3a1 var. 3 (10·8); Dundee (8·6); 3a1 var. 1 (4·3).

(b) 3a1 (2·1); B.A.O.R. (3·1); Taunton (2·1).

ITALY (Northern)

PROFESSOR A. GIOVANARDI – Milan

(1) *Salmonella typhi*

Type distribution by cases: 261.

A (25·67); I+IV (23·75); degraded Vi-strains (13·79); E1 (9·19); C1 (8·82); D1 (8·82); Vi-negative (7·66); D4 (0·77); F1 (0·77); B2 (0·38); N (0·38).

Type distribution by foci: 249.

A (26·90); I+IV (24·89); degraded Vi-strains (14·46); C1 (9·23); Vi-negative (8·04); D1 (7·22); E1 (6·82); D4 (0·81); F1 (0·81); B2 (0·41); N (0·41).

(2) *Salmonella paratyphi B*

Type distribution by cases: 38.

Taunton (44·73); untypable (18·43); B.A.O.R. (13·16); 1 var. 5 (7·90); 1 var. 8 (2·63); 3a1 (2·63); 3b var. 3 (2·63); 3b var. 6 (2·63); Beccles (2·63); Dundee var. 1 (2·63).

Type distribution by foci: 28.

Taunton (35·72); untypable (35·72); 1 var. 5 (3·57); 1 var. 8 (3·57); 3a1 (3·57); 3b. var. 3 (3·57); 3b var. 6 (3·57); Beccles (3·57); B.A.O.R. (3·57); Dundee var. 1 (3·57).

ITALY (Central)

DR D. PARVIS – Pisa

(1) *Salmonella typhi*

Type distribution by cases: 79.

(a) A (24·50); C1 (22·02); D1 (18·86); Vi-negative (16·52); E1 (6·45).

(b) I + IV (5·95); B2 (1·90); degraded Vi-strains (1·90); 46 (1·04); F1 (0·86).

Type distribution by foci: 52.

(a) A (24·48); C1 (20·30); D1 (19·87); Vi-negative (13·46); E1 (7·12); I + IV (5·65).

(b) B2 (3·03); degraded Vi-strains (3·03); 46 (1·56); F1 (1·47).

(2) *Salmonella paratyphi B*

Type distribution by cases: 52.

(a) Taunton (25·89); 3a1 (21·67); 1 (16·05); untypable (14·60); Dundee (6·67); 2 (6·66).

(b) B.A.O.R. (5·88); 3b (1·66); Beccles (1·47).

Type distribution by foci: 39.

(a) Taunton (32·13); 3a1 (25·29); untypable (13·38); 1 (9·81); Dundee (8·03).

(b) 2 (5·35); Beccles (2·08); B.A.O.R. (2·08); 3b (1·78).

NETHERLANDS

National Centre of Utrecht (unsigned report)

(1) *Salmonella typhi*

Number of strains examined: 180.

It is not specified whether the results are given by cases or by foci.

Type distribution:

(a) E1 (26·66); A (19·44); D1 (13·88); C1 (12·22); 46 (7·22); D2 (5·0); D6 (3·33); F1 (2·22); 28 (1·66).

(b) B3 (1·11); D7 (1·11); D 8 (1·11); C9 (0·55); D4 (0·55); D8 (0·55); G1 (0·55); O (0·55); 27 (0·55); 9 (0·55); 35 (0·55); 40 (0·55).

(2) *Salmonella paratyphi B*

Number of strains examined: 215.

(a) Taunton–Kampen (34·41); 1 of F and C (14·88); 3a1 Leewarden (7·88); Jersey (7·88); atypical strains (5·58); 3a1 var. 1–2 (4·65); Dundee (4·65); 3a (4·18); 3a1 Schiedam (3·72); Midwoud (3·25).

(b) 3b (1·86); Sittard (1·86); Beccles–Meppel (1·39); B.A.O.R. (0·93); Q1 (0·93).

(c) 3a var. 1 (0·46); B.T.6 (0·46); Q7 (0·47); P3 (0·46).

NORWAY

DR R. SAXHOLM – Oslo

(1) *Salmonella typhi*

Type distribution by cases: 13.

A: 4 cases. F1: 3. E1: 2. D1: 1. J1: 1. I + IV: 1. Degraded Vi-strains: 1.

(2) *Salmonella paratyphi B*

Type distribution by cases: 21.

1: 9 cases and three foci. Taunton: 4. 3a: 2. 3a1: 2. Untypable: 2. Dundee: 1.

POLAND

PROF. DR Z. BUCZOWSKI – Gdansk

(1) *Salmonella typhi*

Type distribution by cases: 9325.

(*a*) E1a (21·51); F1 (13·33); A (11·34); degraded Vi-strains (11·0); D1 (7·60); Vi-negative (6·87); C1 (5·98); I + IV (4·42); E1b (4·35); 46 (2·85); 28 (1·51).

(*b*) F4 (1·46); D4 (1·20); 40 (1·07); F5 (0·86); D2 (0·77); D9 (0·72); N (0·70); D6 (0·68).

(*c*) D7 (0·30); J1 (0·27); 38 (0·17); B2 (0·16); E7 (0·15); F3 (0·15); E10 (0·08); T (0·07); E3 (0·06); C5 (0·05); C4 (0·04); F7 (0·04); M1 (0·04); C2 (0·03); E4 (0·03); F2 (0·03); 43 (0·03); E9 (0·02); 36 (0·02); C3 (0·01); D5 (0·01); D8 (0·01); 50 (0·01).

Type distribution by foci: 7402.

(*a*) E1a (21·57); F1 (13·35); degraded Vi-strains (11·55); A (11·11); D1 (7·95); Vi-negative (7·50); I + IV (4·91); C1 (4·36); E1b (4·07); 46 (2·85).

(*b*) F4 (1·62); D4 (1·39); 40 (1·17); D9 (1·04); F5 (0·89); D6 (0·78); N (0·78); 28 (0·72); D2 (0·52).

(*c*) J1 (0·31); B2 (0·20); 38 (0·20); F3 (0·18); D7 (0·17); E7 (0·12); T (0·12); E10 (0·10); E3 (0·07); C4 (0·05); C5 (0·05); F7 (0·05); M1 (0·05); C2 (0·04); E4 (0·04); E9 (0·02); F2 (0·02); 36 (0·02); 43 (0·02); C3 (0·01); D5 (0·01); D8 (0·01); 50 (0·01).

(2) *Salmonella paratyphi A*

Type distribution by cases and by foci: 36.

(*a*) 1 (75·02); 4 (16·66).

(*b*) Untypable (5·55); 3 (2·77).

(3) *Salmonella paratyphi B*

Type distribution by cases: 1670.

(*a*) Taunton (35·07); 1 (20·08); B.A.O.R. (14·09); 3a1 var. 1–2 (9·16); 3a (5·98); 3a1 (4·85).

(*b*) Untypable (4·49); Dundee (1·79); Beccles (1·61); 3b (1·49); Jersey (1·01).

(*c*) 3b var. 1 (0·17); 3a var. 1 (0·11); 3a var. 2 (0·05); 3a var. 4 (0·05).

Type distribution by foci: 1332.

(*a*) Taunton (36·17); 1 (19·06); B.A.O.R. (11·48); 3a1 var. 1–2 (10·93); 3a1 (5·78); untypable (5·10).

(*b*) 3a (3·67); Beccles (2·10); Dundee (2·10); 3b (1·95); Jersey (1·12).

(*c*) 3b var. 1 (0·22); 3a var. 1 (0·15); 3a var. 2 (0·07); 3a var. 4 (0·07).

PORTUGAL

Dr A. C. Sampaio – Lisbon

(1) *Salmonella typhi*

Type distribution by cases: 81.

(a) E1 (38·27); A (24·69); B3 (11·11); 46 (7·41); D1 (6·17).

(b) Degraded Vi-strains (6·17); I + IV (3·70); Vi-negative (2·47).

Type distribution by foci: 37.

(a) A (27·03); E1 (13·51); 46 (13·51); degraded Vi-strains (13·51); B3 (8·81); D1 (8·81).

(b) I + IV (5·41); Vi-negative (5·41).

(2) *Salmonella paratyphi B*

3 strains. 3a var. 4: 2 strains. Untypable: 1 strain.

ROMANIA

First Prof. N. Nestoresco (now deceased) then
Dr M. Popovici – Bucharest

(1) *Salmonella typhi*

Type distribution by cases: 2397.

(a) A (22·24); E1 (17·33); F1 (15·80); degraded Vi-strains (9·39); D9 (7·92); D1 (7·14); I + IV (7·12); Vi-negative (4·27).

(b) C1 (3·98); C4 (0·74); F5 (0·73); 38 (0·64); 46 (0·58).

(c) D6 (0·42); C2 (0·30); F3 (0·21); 48 (0·18); D4 (0·16); 40 (0·16); 28 (0·12); B2 (0·11); C5 (0·10); D2 (0·09); E10 (0·05); F4 (0·05); T (0·05); K1 (0·03); M1 (0·03); K2 (0·03).

Type distribution by foci: 1589.

(a) A (24·98); E1 (15·72); F1 (14·17); degraded Vi-strains (9·55); I + IV (7·42); D1 (6·84); D9 (6·75); Vi-negative (5·16).

(b) C1 (4·32); C4 (0·88); 38 (0·87); F5 (0·72); 46 (0·54).

(c) D6 (0·44); F3 (0·25); C2 (0·21); 40 (0·19); D2 (0·15); 28 (0·13); C5 (0·10); B2 (0·08); D4 (0·08); E10 (0·08); F4 (0·08); T (0·08); K1 (0·05); K2 (0·05); M1 (0·05); 48 (0·05).

(2) *Salmonella paratyphi B*

Type distribution by cases: 68, and by foci: 68.

(a) Atypical (23·52); untypable (16·17); Taunton (14·70); 1 (11·76); B.A.O.R. (10·29); 1 var. 4 (7·35); 3a1 (4·41); Dundee (4·41).

(b) 3a var. 4 (2·94); 3a var. 1 (1·47); 3a var. 6 (1·47); 3a var. 4 (1·47).

SPAIN

Dr J. Ruiz Merino – Madrid

(1) *Salmonella typhi*

Type distribution by cases: 44.

(a) E1 (29·54); A (20·45); D9 (20·45); 46 (6·81); D1 (4·54); T (4·54); I + IV (4·54).

(b) C1 (2·27); D5 (2·27); D6 (2·27); N (2·27).

Type distribution by foci: 30.

(a) E1 (43·33); A (13·33); D1 (6·66); T (6·66); 46 (6·66); I+IV (6·66).

(b) C1 (3·33); D5 (3·33); D6 (3·33); D9 (3·33); N (3·33).

(2) *Salmonella paratyphi B* (2 cases).

B.A.O.R. (2 cases, 2 foci).

SWEDEN
DR L. O. KALLINGS – Stockholm

(1) *Salmonella typhi*

Type distribution by cases: 54.

(a) E1 (24); A (15); F1 (13); C1 (11); 46 (9); degraded Vi-strains (9); E3 (4); NST (4).

(b) D1 (2); D6 (2); J1 (2); N (2); 32 (2).

Type distribution by foci: 52.

(a) E1 (25); A (15); C1 (12); F1 (10); 46 (10); degraded Vi-strains (10); E3 (4); NST (4).

(b) D1 (2); D6 (2); J1 (2); N (2); 32 (2).

(2) *Salmonella paratyphi B*

Type distribution by cases: 276.

Taunton (53); Dundee (11); 3a1 var. 4 (7); NST (5); 1 (4); 1 var. 2 (4); Beccles (3); NT (3); 1 var. 1 (1); 1 var. 6 (1); 1 var. 8 (1); 3a (1); 3a1 var. 1 (1); 3b (1); Jersey (1); B.A.O.R. (1); Dundee var. 1 (1); 1 var. 3 (< 1); 1 var. 4 (< 1); 3a var. 4 (< 1); 3a1 var. 2 ? (< 1); 3b var. 1 (< 1); 3b var. 6 (< 1); Taunton var. 1 (< 1).

Type distribution by foci: 210.

Taunton (50); Dundee (13); NST (7); 1 (4); 1 var. 2 (3); Beccles (3); NT (3); 3a1 var. 1 (2); B.A.O.R. (2); 1 var. 1 (1); 1 var. 6 (1); 3a (1); 3a1 (1); 3a1 var. 4 (1); 3b (1); Jersey (1); Dundee var. 1 (1); 1 var. 3 (< 1); 1 var. 4 (< 1); 1 var. 8 (< 1); 3a var. 4 (< 1); 3a1 var. 2 ? (< 1); 3b var. 1 (< 1); 3b var. 6 (< 1); Taunton var. 1 (< 1).

SWITZERLAND
PROFESSOR H. FEY – Berne

(1) *Salmonella typhi*

Type distribution by cases: 547.

(a) C1 (26·87); A and degraded A (23·94); E1 (12·43); B2 (5·85); untypable (5·85); D1 (5·30); C4 (4·57); N (3·47); 46 (2·55).

(b) C3 (1·46); D4 (1·46); group B (1·27); C2 (1·09); 38 (0·54).

(c) Group D (0·36); D2 (0·36); E4 (0·36); E7 (0·36); G1 (0·36); K2 (0·36); C5 (0·18); C7 (0·18); E3 (0·18); F4 (0·18); Vi-negative (0·18); F1 (0·18).

(2) *Salmonella paratyphi B*

Type distribution by cases: 339.

(a) Taunton (30·67); untypable (20·35); 1 var. 4 (8·25); Dundee (5·84); B.A.O.R. (5·01); 1 (4·71); group 1 (3·83); 3a1 var. 1 (2·65); Beccles var. 3 (2·35); Beccles (2·06); 3a1 var. 4 (1·76); 3a1 (1·47).

(b) 2 (1·17); 3a (1·17); Taunton var. 1 (1·17); 3 (0·88); 1010 (0·88); 3a var. 2 (0·58); Beccles var. 2 (0·58); Jersey (0·58); Worksop (0·58).

(c) 1 var. 1 (0·29); 1 var. 2 (0·29); 1 var. 11 (0·29); 3a var. 4 (0·29); 3a var. 7 (0·29); 3b (0·29); 3b var. 1 (0·29); 3b var. ? (0·29); Beccles var. 1 (0·29); Beccles var. 5 (0·29); Beccles n.n.t (0·29).

UNITED KINGDOM
DR E. S. ANDERSON – London

(1) *Salmonella typhi*

Type distribution by cases (267) of U.K. origin.

(a) E1 (17·9); A (15·8); Degraded Vi-strains (14·4); C1 (11·5); O (9·4); 46 (8·6); I+IV (7·2); Vi-negative (4·4); D1 (3·4).

(b) K1 (1·8); F1 (1·4); B2 (0·8); D6 (0·5).

(c) D4 (0·4); D6 (0·4); F4 (0·4); N (0·4); 45 (0·4); B1 (0·3); D2 (0·3); 28 (0·3).

Type distribution by foci (158) of U.K. origin

(a) E1 (17·3); A (17·1); Degraded Vi-strains (12·00); C1 (9·8); 46 (8·1); I+IV (8·1); O (7·2); Vi-negative (5·9); D1 (3·2); K1 (2·9).

(b) F1 (2·5); D6 (0·8); D4 (0·6); D10 (0·6); F4 (0·6); N (0·6); 45 (0·6); B1 (0·5); B2 (0·5); D2 (0·5); 28 (0·5).

Type distribution by cases (422) of foreign origin.

(a) E1 (19·4); Degraded Vi-strains (14·1); I+IV (12·3); A (10·7); D1 (10·0); C1 (6·1); O (5·6); 46 (3·3); 45 (2·1); 40 (2·00); C4 (1·5); F1 (1·5); Vi-negative (1·2).

(b) D2 (1·1); 28 (1·1); C5 (1·0); G1 (1·0); N+D1 (0·8); 38 (0·8); K1 (0·7); N (0·5); T (0·5).

(c) C3 (0·3); D5 (0·3); D6 (0·3); 32 (0·3); 34 (0·3); B2 (0·2); F4 (0·2); D2 (0·2); G2 (0·2); 42 (0·2).

Type distribution by foci (223) of foreign origin.

(a) E1 (14·9); degraded Vi-strains (14·1); A (11·5); I+IV (9·2); D1 (8·3); C1 (5·9); 46 (5·0); O (4·4); 40 (2·8); Vi-negative (2·3); G1 (2·1); C4 (2·0); 28 (2·0); F1 (1·8); 38 (1·7); 45 (1·7).

(b) T (1·2); C5 (1·1); N+D1 (1·1); K1 (0·9); N (0·9); D2 (0·8); C3 (0·6); D6 (0·6); D5 (0·5); 32 (0·5); 34 (0·5).

(c) B2 (0·4); F4 (0·4); G2 (0·4); 42 (0·4).

(2) *Salmonella paratyphi B*

Type distribution by cases (484) of U.K. origin.

(a) 1 (23·1); Taunton (14·0); Battersea (11·0); 3a (9·7); untypable (7·9); Dundee (4·6); Dundee var. 1 (4·0); 1 var. 1 (3·2); Worksop (3·2); 3a1 var. 1 (2·3); Beccles (2·2); 1 var. 9 (1·9); B.A.O.R. (1·9); 3a var. 2 (1·5).

(b) 3b var. 3 (1·4); Scarborough (1·2); 1 var. 4 (1·0); Jersey (0·8); 1 var. 2 (0·6); 1 var. 3 (0·6); 50 (0·5).

(c) 2 (0·4); 3b (0·4); 2 var. 1 (0·3); 3a var. 6 (0·3); 3a1 (0·3); 3b var. 6 (0·3); Taunton var. 1 (0·3); untypable r.d.n.c. (0·3); Beccles var. 3 (0·2); Beccles var. 5 (0·2); Beccles var. 6 (0·2); Jersey var. 3 (0·2).

Type distribution by foci (259) of U.K. origin.

(*a*) 1 (23·9); Taunton (15·2); 3a (8·7); Dundee (8·2); untypable (6·2); Battersea (5·6); 1 var. 1 (4·8); Dundee var. 1 (4·8); Beccles (3·6); 3a1 var. 1 (2·3); 1 var. 4 (2·0); Worksop (1·8); 3a var. 2 (1·8); 50 (0·9); Jersey (0·9).

(*b*) 1 var. 2 (0·8); 1 var. 3 (0·8); 2 (0·8); 3b (0·8); untypable r.d.n.c. (0·8); 2 var. 1 (0·7); B.A.O.R. (0·7).

(*c*) 1 var. 9 (0·4); 3a var. 6 (0·4); 3a1 (0·4); 3b var. 3 (0·4); Beccles var. 5 (0·4); Jersey var. 3 (0·4); Scarborough (0·4); Taunton var. 1 (0·4); 3b var. 6 (0·3); Beccles var. 3 (0·3); Beccles var. 6 (0·3).

Type distribution by cases (195) of foreign origin.

(*a*) Taunton (45·0); Dundee (14·4); 1 (8·0); untypable (7·8); 3a var. 4 (4·0); Dundee var. 1 (3·2); 3a1 (2·7); 3a1 var. 1 (2·5); B.A.O.R. (2·4).

(*b*) Beccles var. 3 (1·9); Beccles (1·6); 3b var. 9 (1·5); Battersea (1·5); 1 var. 4 (1·0); Jersey var. 2 (0·8); Jersey (0·6); Taunton var. 1 (0·6); untypable r.d.n.c. (0·5).

Type distribution by foci (103) of foreign origin.

(*a*) Taunton (37·5); Dundee (12·8); untypable (9·8); Dundee var. 1 (5·8); 3a var. 4 (4·9); 3a1 var. 1 (4·3); 1 (4·0); B.A.O.R. (3·7); Beccles (3·4); 3a1 (2·8); Beccles var. 3 (2·8).

(*b*) 1 var. 4 (1·8); 3b var. 9 (1·2); Battersea (1·2); untypable r.d.n.c. (1·2); Jersey (1·1); Taunton var. 1 (1·1); Jersey var. 2 (0·6).

YUGOSLAVIA

Dr P. Tomasic, then Dr A. Z. Dragas – Zagreb

(1) *Salmonella typhi*

Type distribution by cases: 1257.

(*a*) A (43·44); D1 (18·38); E1a (8·67); C1 (6·6); Vi-negative (6·04); F1 (5·96).

(*b*) Degraded Vi-strains (2·39); 28 (2·07); 46 (1·43); C5 (1·35); E10 (0·87); I + IV (0·8); C2 (0·64).

(*c*) D9 (0·40); B2 (0·24); F4 (0·16); B1 (0·08); C9 (0·08); D2 (0·08); E2 (0·08); J4 (0·08); N (0·08); 34 (0·08).

Type distribution by foci: 672.

(*a*) A (46·58); E1a (11·46); F1 (9·67); Vi-negative (8·78); C1 (6·55); D1 (5·35).

(*b*) Degraded Vi-strains (3·57); 46 (2·08); C5 (1·04); 28 (1·04); I + IV (0·89); E10 (0·74).

(*c*) C2 (0·45); B2 (0·30); D9 (0·30); B1 (0·15); C9 (0·15); D2 (0·15); D9 (0·15); E2 (0·15); F4 (0·15); J4 (0·15); N (0·15); 34 (0·15).

(2) *Salmonella paratyphi B*

Type distribution by cases: 201.

(*a*) Taunton (69·65); 3a (11·45); 1 (5·47); Jersey (3·48); Dundee (3·48).

(*b*) 3a1 (1·49); 3b (1·49); Beccles (1·49); untypable (1·00); 1 var. 3 (0·50); 1 var. 4 (0·50).

Type distribution by foci: 165.

(*a*) Taunton (65·45); 3a (11·52); 1 (6·66); Jersey (4·24); Dundee (4·24).

(*b*) 3a1 (1·82); 3b (1·82); Beccles (1·82); untypable (1·21); 1 var. 3 (0·61); 1 var. 4 (0·61).

<div align="center">DR NADA STOSIC – Belgrade</div>

(1) *Salmonella typhi*

Type distribution by cases: 541.

(*a*) A (24·7); E1 (14·2); I + IV (12·2); D4 (11·8); D1 (9·2); 28 (7·9); degraded Vi-strains (5·3); F1 (4·8).

(*b*) C1 (2·7); T (2·2); Vi-negative (2·0); D6 (1·6).

(*c*) C4 (0·3); C5 (0·3); 25 (0·1).

(2) *Salmonella paratyphi B*

Type distribution by cases: 172.

(*a*) Taunton (80·4); 3a (9·8).

(*b*) Beccles (9·3).

(*c*) 3a1 (0·4).

<div align="center">DISCUSSION</div>

A. As in previous reports (Anderson, 1961; Nicolle, 1961*a, b*) important differences have been noted in the distribution of Vi-phage types and untypable groups of *Salmonella typhi* during the period 1966–1969, depending on the geographical origins of the strains considered.

Unfortunately, while a large number of strains (several hundred, and sometimes several thousand) were studied in many countries of Europe, Africa (North and South) and America (U.S.A., Canada), in many parts of the world, in contrast, the number was not representative of the total incidence of *S. typhi*. Consequently, any comparison of the distribution of phage types suffers from a fundamental fault; the number of phage types and varieties identified by a particularly busy centre will be high, whereas in another country, where only a small proportion of strains of *S. typhi* are sent to the National Centre for Enteric Phage Typing, the number of phage types may appear to be much lower.

For the comparison to be valid, each country should examine an equivalent proportion of strains or foci; such was not the case.

B. In spite of these serious objections, as we have already pointed out in previous reports (Anderson, 1961; Nicolle, 1961*a, b*), the Vi-phage types of *S. typhi* can be divided into groups according to their geographical distribution.

(1) Cosmopolitan phage types, generally the commonest types in Europe: A, B2, C1, D1, E1, F1, N, T, 28, 46, etc., and the three groups of untypable strains: I + IV; degraded Vi-strains; Vi-negative.

(2) Phage types frequently found in North Africa (Morocco, Algeria, Tunisia, Egypt) but less common or even non-existent in other parts of the world: L1, L2, 40, 42, etc.

(3) Phage types common in Black Africa and rare, often non-existent, in Europe

and in most parts of the world: C2, C4, D6, Central African variety of type C1, etc.

(4) Phage types especially common in countries bordered by the Indian Ocean: G1, etc.

(5) Phage types common or present only in the Far East, and rare or non-existent in most other parts of the world: C3, C5, D2, D4, E2, E3, E4, E7, E9, E10, G3, G5, J3, J5, M2, M3, M4 and, above all, type 37 and some I + IV groups.

(6) Phage type M1, rare or non-existent in Europe and Africa but, in contrast, common in the Far East, in the countries of the West coast of the Pacific Ocean and also found in Australia.

(7) Phage types present almost exclusively in America, especially Latin America: 26, 35, 38, etc.

To sum up, despite all the intermingling of strains of the typhoid bacillus caused by population migrations, wars, invasions, etc., important differences in the geographical distribution of phage types are still evident. Some types are cosmo-politan or semi-cosmopolitan. Others appear more or less limited to one area or to a group of neighbouring areas. It seems that the typhoid bacillus, which was initially homogeneous, has diversified in various regions of the world, and as if certain varieties, carried out of their original habitat, have reached distant regions, while others have extended only to a limited degree from their original territory.

From the phage typing of *S. typhi* we can therefore learn about the present distri-bution of phage types in different parts of the world, the changing distribution of phage types which is taking place, and the causes of these variations.

The same conclusions, perhaps not quite so clearly defined, can be drawn from the phage-typing studies of *S. paratyphi B.*

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Salmonella serotypes isolated from tortoises and frogs in Istanbul

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SUMMARY

The incidence of *Salmonella* in tortoises and frogs captured in Istanbul was studied. From 31 out of 40 tortoises (77·5 %) and from 16 out of 311 frogs (5·1 %) salmonella strains belonging to 18 and 14 serotypes respectively were isolated.

INTRODUCTION

There are several reports on the isolation of *Salmonella* serotypes from cold-blooded animals. Although many articles concern the salmonella strains in tortoises, snakes and lizards (LeMinor, Fife & Edwards, 1958; Özek, Çetin, Anğ & Töreci, 1965; de Hammel & McInnes, 1971), there are only a few reports on the presence of salmonellas in frogs (Pantaléon & Rosset, 1964; Desmet-Paix, Lambion, Van Oye & Veslemans, 1968; Özek *et al.* 1969). The present paper records the serotypes and incidence of salmonellas in tortoises and frogs captured in the suburbs of Istanbul.

MATERIALS AND METHODS

The stools obtained every day from each of the 40 tortoises, which were kept separately in isolated boxes, were cultured.

Liver, spleen, heart, kidney, leg muscles and intestinal contents of each of 311 frogs were examined separately for the presence of salmonella strains. The frogs, under ether narcosis, were fixed on sterile wooden plates. After cleaning the skin with alcohol, the frogs were dissected with sterile tools. The above mentioned parts of the body were removed, cut into small pieces and inoculated into tubes containing Mueller–Kaufmann and selenite F broths. After 48 hr. incubation subcultures were made on Endo and deoxycholate citrate media. The identification of suspected colonies on these media was carried out with the usual bacteriological and serological techniques.

RESULTS

From 31 out of 40 tortoises (77·5 %) 17 *Salmonella* serotypes and two *Arizona* strains were grown. Table 1 shows the distribution of these among the tortoises. Four different serotypes were grown from each of two tortoises, three serotypes (one an *Arizona* type) from one, and two serotypes (one *Arizona*) from 17 tortoises.

Eighteen of the 31 tortoises from which different salmonella serotypes were

Ö. ANǦ, Ö. ÖZEK, E. T. ÇETİN AND K. TÖRECİ

Table 1. Salmonella *serotypes isolated from tortoises*

Tortoise no.	S. abony	S. abortus bovis	S. arizona	S. boecker	S. canastel	S. charity	S. clifton	S. halle	S. hermannsverder	S. hofit	S. hvittingfoss	S. iramu	S. java	S. kottbus	S. mikawasima	S. nashua	S. potsdam	S. softa
1	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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5	.	.	.	.	.	+	.	+	.	.	.	.	.	.	.	.	.	.
6	.	.	.	.	.	.	.	.	.	.	.	+	.	+	.	+	+	.
7	.	+	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.
8	+	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
9	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.
10	+	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
11	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	+	.
12	+	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	+	+
13	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
14	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.
15	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.
16	+	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
17	+	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
18	+	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.
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23	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+
26	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.
27	+	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.
28	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
29	.	.	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.
30	+	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.
31	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.
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35	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.
37	+	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.

isolated in our investigation were dissected under ether anaesthesia and their bloods were taken. Autopsies were performed and cultures were made from their blood and organs. The media into which their blood and interior organs were added remained sterile.

From 16 out of 311 frogs (5·1 %) 15 *Salmonella* strains belonging to eleven serotypes, and three *Arizona* serotypes, one of each, were grown. The distribution of these in the organs of individual frogs is shown in Table 2.

Table 2. Salmonella *serotypes isolated from frogs and their sites of isolation*

Frog no.	Site of isolation	Salmonella serotype
17	Liver	*S. bovis-morbificans*
48	Liver	*S. arizona* (47:c:e, n, x, z_{15})
112	Liver	*S. newport*
113	Liver and leg muscles	*S. newport*
118	Liver	*S. arizona* (61:r:z_{53})
120	Leg muscles and intestinal contents	*S. hvittingfoss*
153	Intestinal contents	*S. abony* var. *haifa*
154	Liver	*S. abony, S. kottbus, S. istanbul*
159	Liver	*S. abony* var. *haifa*
164	Liver	*S. arizona* (61:i:z_{53})
196	Liver	*S. mikawasima*
204	Liver	*S. hofit*
232	Liver	*S. richmond*
235	Liver	*S. boecker*
241	Liver	*S. hofit*
267	Liver	*S. kottbus*

DISCUSSION

Salmonella serotypes were first encountered in tortoises in 1946 (Boycott, Taylor & Douglas, 1953). A number of authors stress the potential importance of tortoises namely pet turtles as carriers of salmonellas. All kinds of tortoises are not salmonella carriers in the same proportion. For example, Vincent, Neel & LeMinor (1960) found the incidence to be 78 % among the *Testuda graeca* captured in Maroc, Dimow, Wesselinof & Rohde (1961) 56 % among tortoises in Bulgaria, and Bövre & Sandbu (1959) 81·8 % in Norway among the tortoises brought from the Mediterranian countries.

The presence of salmonellas in frogs was established by some reports (Pantaléon & Rosset, 1964; Desmet-Paix *et al.* 1968). This finding may be especially important for human health in countries where frog meat is consumed. In Canada, several Salmonella serotypes were isolated from imported and native frogs legs. In France Pantaléon & Rosset (1964) found 31 out of 164 specimens of imported frog muscles to contain one or two of 13 different serotypes. Of these, *S. newport* and *S. hvitting-foss* were also encountered in our study. In the present work, the specimens were taken from each living animal to gain an accurate opinion about the percentage of salmonella organisms in frogs. This also prevented contamination from other sources which might occur in the course of the preparation of frogs' leg muscles as canned food, which were investigated by other authors. Sixteen of 311 frogs (5·1 %) were found to contain salmonella strains. One of the frogs had three serotypes in the liver. In two frogs, two different specimens gave positive cultures for the same serotypes. The presence of salmonella strains only in the liver in thirteen instances suggests that the first organ to be examined is the liver, but the reason for this finding is obscure.

Nearly all of the *Salmonella* serotypes isolated from tortoises and frogs in this study were encountered in Turkey for the first time.

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An epidemic of influenza on Tristan da Cunha

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SUMMARY

Respiratory disease on Tristan da Cunha has been observed since the islanders returned in 1962. An epidemic of unprecedented severity occurred in the winter of 1971 and involved 273 (96%) of 284 islanders, 92 of whom had two attacks.

The epidemic was apparently introduced by the *Tristania*.

The illness of both first and second attacks ranged from mild to severe but there were some differences. There were two deaths, both in elderly persons with chronic chest disease and heart failure. Serological evidence suggests that this was due to influenza A2 of the Hong Kong serotype H_3N_2.

INTRODUCTION

Tristan da Cunha is a remote volcanic island in the South Atlantic. The isolated population has for many years been subject to epidemics of common colds which follow the arrival of supply ships from Cape Town (Woolley, 1946; Shibli, Gooch, Lewis & Tyrrell, 1971). It is many years since an epidemic of influenza-like illness occurred there, but one occurred in the winter of 1971 and is reported here because of some unusual features.

MATERIALS AND METHODS

The clinical records were drawn from the notes taken during the regular work of the practice, since the previous regular respiratory disease survey had been discontinued. All but three houses were visited and this gave opportunities to record milder illnesses as well as those requiring medical attention; further enquiries were made through two island nurses. As has been found before, it was possible to obtain almost complete records of illness on Tristan. The practice population consisted of 284 islanders and 20 expatriates, but the figures presented are for the islanders only.

Laboratory studies

Sera were collected and separated on the island, and sent by sea in refrigeration to Cape Town and thence by air to Britain. They were tested by haemagglutination-inhibition tests as described previously (Tyrrell, Peto & King, 1967) and by complement fixation tests against standard respiratory virus antigens.

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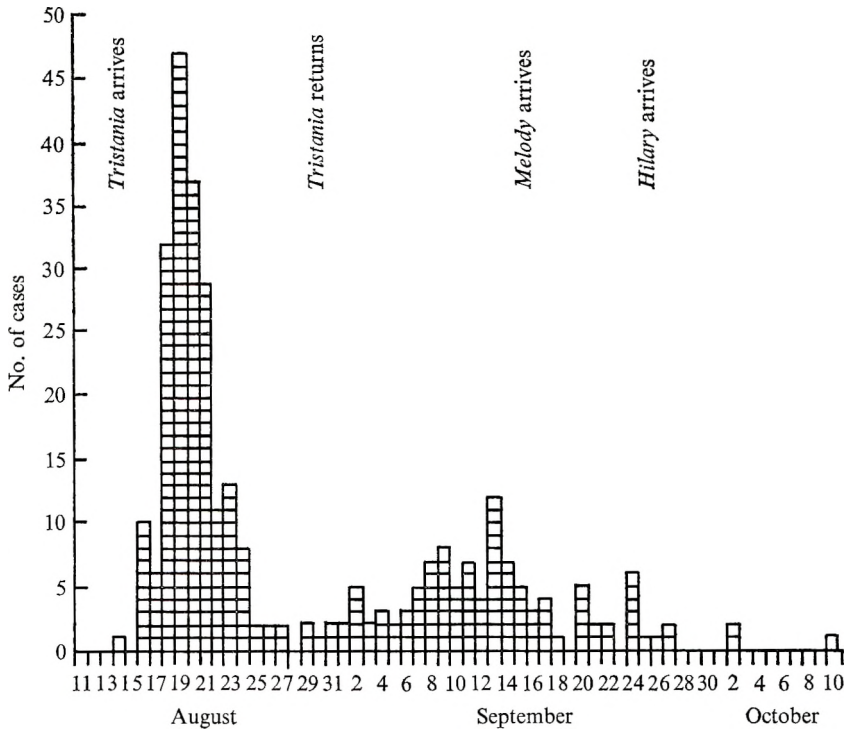
Fig. 1. The course of the epidemic. Each square indicates the day of onset of a separate case of illness. I or II in the 312 cases in which it was known for certain. *Tristania* arrives 13 August, disembarks five islanders and embarks JY. *Tristania* returns (from local fishing) 30 August, and lands JY and MR. *Melody*, fishing vessel ex-Cape Town, lands mail 16 September. *Hilary*, fishing vessel ex-Cape Town, lands mail 26 September.

RESULTS

On 13 August 1971, the fishing vessel *Tristania* landed five islanders after an 8-day voyage from Cape Town. The islanders had either been receiving medical treatment or had been on holiday in Cape Town. Three of them developed acute respiratory disease (ARD) in the course of the voyage and two had a similar illness immediately after landing. Various family gatherings welcomed their disembarkation. In the next few days all those who had been in contact with the returning islanders developed ARD which spread rapidly throughout the whole island population. The illness was similar in both expatriates and islanders, except that it tended to be milder in the former, particularly in those who had recently arrived.

At the beginning of the fourth week of the epidemic it became clear that some islanders were developing second attacks of respiratory disease and a second peak of new cases was recorded. The last new case was recorded on 10 October.

The origin and course of the epidemic

Fig. 1 shows the number of new cases beginning on each day and the dates of arrival of ships. Only those cases of which the date of onset was known precisely were included, i.e. 312 of the total of 365.

On arriving the *Tristania* disembarked five islanders and, for 2 days only, an English marine biologist (MR). During these 2 days there was some superficial contact between the ship's crew and islanders while supplies were unloaded. After 2 days the *Tristania* departed to fish in local waters, taking on board the expatriate manager of the crawfish freezing plant (JY) and MR. During the 15 days between the first and second arrival of the *Tristania* the ship had no outside contact. There was no respiratory disease on board the ship during this period – indeed the only ascertainable illness whatsoever among the crew on board was a mild cold which the captain suffered during the first 2 days out from Cape Town on the original voyage. Therefore, from the departure of the *Tristania* from Cape Town on 5 August until the arrival of the fishing vessel *Melody* on 16 September, the island and the *Tristania* together constituted a closed epidemiological group. On 30 August the *Tristania* called briefly to disembark JY and MR and take on water. Four days later JY developed a mild cold.

Ninety-six per cent of the Tristan islanders (273 out of 284) developed one episode of respiratory disease; 33 % (92) developed a second episode of respiratory disease. It is difficult to analyse the data without making epidemiological assumptions, but the least objectionable method seems to be to classify the illness according to whether

(a) it was the only episode, or the first of two episodes (illness I);

(b) it was the second of two episodes (illness II).

A few patients classified as having illness I developed it after other patients developed illness II. The first case of a second episode of respiratory disease occurred at the end of the third week of the epidemic, by which time 215 islanders had been infected with what is by definition illness I. This figure (215) represents 76 % of the island population at risk and 97 % of those who suffered from at least one illness.

Clinical features of the illnesses

In the majority of cases (85 % of those affected) illness I consisted of a typical influenza-like illness of at least moderate severity. The onset was sudden, with fever ranging from 100–104° F. presaged by shivering attacks or, in a few cases, actual rigors, and generalized aching pains in the back and legs. There was often prostrating headache with vomiting for 24 hr. and this occurred more often in women than in men. Cough was marked after the first day and tended to persist, even in the absence of secondary infection, for up to 3 weeks. Fever and malaise lasted an average of 3 days. In younger children (aged below 10 years) and babies the illness was usually comparatively mild, lasting only 2 days and without respiratory sequelae. In some adult islanders also the illness was quite mild, with fever not exceeding 100° F. and only transient cough and malaise. Coryza was not prominent. Sore throats were common, usually after the acute stage had passed.

Table 1 shows the extent and severity of illness I amongst the Tristan islanders. In mild illness the patient was not confined to the house or to bed for more than a day. Moderate describes what would be accepted by both lay and medical people as a typical case of influenza-like illness without major complications. Severe

Table 1. *The clinical features of the illnesses seen*

	Observations on	
	Illness I	Illness II
Grade of illness in islanders		
None	11 (4)	192 (67)
Mild	41 (14)	42 (15)
Moderate	189 (67)	36 (13)
Severe	43 (15)	14 (5)
Total	284 (100)	284 (100)
Complications in patients		
Chest infection	36 (13)	21 (23)
Bronchospasm	23 (8)	11 (12)
Pleural pain	12 (4)	6 (6)
Haemoptysis	4 (1·5)	3 (3)
Cardiovascular	1 (0·3)	0 (0)
Otitis media	7 (2·6)	2 (2)
Total	273 (100)	92 (100)

N.B. Figures in brackets are percentages.

illness was characterized by severe secondary infection, or other complications such as persistent pleuritic pain, severe bronchospasm, cardiovascular involvement, or by an unusual degree of prostration and fever.

The incidence of complications in illness I is also shown. Secondary bacterial chest infection was diagnosed by persistent fever in association with the expectoration of purulent sputum and clinical or radiological signs in the chest.

Only 54 % of the 92 islanders who developed a second illness, illness II, had typical influenza-like moderate illness and less than half of the whole population was affected at all by it (Table 1). Nevertheless, no less than 21 islanders developed two episodes of influenza-like illness.

The second episode of viral respiratory disease was readily distinguished from relapse due to secondary infection. It was usually associated with a blocked or running nose. Fever was less and of more gradual onset than in first episodes. There were invariably coryzal symptoms (which indeed were the only symptoms in about one-third of those affected); involvement of the lower respiratory tract followed later.

Table 1 shows the incidence of complications in illness II, which is similar to that after the first illness, but relatively more secondary chest infections followed illness II. There was one death attributable to illness I and one to the effects of illness II; both were in elderly islanders with chronic respiratory disability and heart failure.

Serological results

The sera were not obtained until the epidemic was almost over and the details of the patients and the timing of the specimens are shown in Table 2. In spite of the small numbers several points are clear. First, the islanders' sera contained significant levels of antibody against Hong Kong influenza A2 haemagglutinin. The last known exposure to influenza A2 was by vaccination in England in 1961

Table 2. *Results of antibody titrations on sera from islanders with moderate or severe illnesses*

Patient	Date of illness		Date of serum	Titre of serum against influenza			
				Influenza A	A2/HK	Influenza B	B/1970
	A	B		CFT	HI	CFT	HI
82	20. viii. 71	10. ix. 71	10. ix. 71	0	1280	0	0
			9. x. 71	0	1280	0	0
9	17. viii. 71	—	8. x. 71	320	320	0	10
111	17. viii. 71	—	8. x. 71	80	1280	0	10
248	19. viii. 71	—	8. x. 71	>320	>5120	0	20
10	20. viii. 71	—	8. x. 71	320	640	0	0
112	20. viii. 71	—	8. x. 71	>320	1280	0	5
241	20. viii. 71	—	8. x. 71	0	1280	0	0
194	22. viii. 71	—	9. x. 71	320	320	0	0
183	24. viii. 71	9. ix. 71	8. x. 71	0	320	0	5
37	2. ix. 71	—	8. x. 71	>320	320	0	0
JPM	10. ix. 71	—	10. ix. 71	0	320	20	0
Expatriate			8. x. 71	20	640	20	0

0 = <5 in HI, <10 in CFT.

and during a subsequent epidemic, long before the Hong Kong variant appeared. There were titres of HI antibodies against A2/Eng/12/64 some of which were higher than those against A2/HK, but these were presumably recalled. Recent infection with influenza A is suggested strongly by the presence of high titres of antibody against the soluble (nucleoprotein) antigen (Table 2) and by a rising titre (from <10 to 20) in JPM, the one expatriate who was bled. Furthermore, there was evidence of influenza A2 infection in those who became ill for the first time both in the first and the second wave of infection. The only CFT antibodies against influenza B were found in the serum of the expatriate and these did not change, and there were only occasional low titres in complement-fixation tests against measles, respiratory syncytial virus, herpes simplex and parainfluenza 3.

DISCUSSION

It is difficult to understand why there were two distinct waves of illness and why one third of the islanders developed two separate illnesses, both obviously viral in origin, in the course of one epidemic. This is the first epidemic in which islanders have been observed to be ill twice, although some anomalies in the epidemic curves have been interpreted as being possibly due to epidemics caused by two agents (Hammond & Tyrrell, 1971). There are three possible explanations for this.

The first is that two separate viral agents were introduced onto the island when the *Tristania* first arrived on 13 August. Each pursued its course through the community at a different rate and produced, therefore, two waves of illness. Each agent must have been capable of causing respiratory disease of varying severity, since some islanders had two mild illnesses and some two episodes of typical or

even severe influenza-like illness. If this explanation is correct, then it is only possible to say that 96 % of the population was infected by one or the other virus, and 33 % by both. In other words, illness I could have been due in some cases to either virus (i) or (ii) and conversely illness II could have been due either to virus (ii) or (i). However, the time factor, the necessarily different infectivity rates and the explosive spread of the first outbreak of ARD suggest that the degree of overlap is small. Even accepting this explanation, it is probable that the patients classified as having illness I are a fairly homogeneous group, epidemiologically speaking.

The second possible explanation is that one virus was introduced when the *Tristania* first arrived and a second virus was introduced when JY and MR disembarked from the *Tristania* on her second call on 30 August, after 15 days at sea. JY developed a mild cold 4 days after coming ashore (3 September). Against this, previous records on Tristan suggest that ships more than 3 weeks out of port are unlikely to initiate epidemics of respiratory disease (Shibli et al. 1971). There had been no respiratory disease among the crew on board the *Tristania* since 10 August. There was time for JY to acquire a cold on the island rather than on the ship.

The third possible explanation is that one virus introduced on 13 August was responsible for the whole epidemic. Antigenic change in the virus may have occurred, allowing second infections, or some patients may not have become fully immune and have suffered a recrudescence of infection or been reinfected from another patient. The epidemiological and laboratory findings lead us to accept the third explanation.

There is clinical and serological evidence that almost all the islanders were infected with influenza A in 1954 (Taylor-Robinson & Tyrrell, 1963). The very high incidence on both occasions may have been due to some lack of immunity and the close-knit society which facilitated transmission. Double illnesses, some apparently due to virus infection, were reported by many in the 1918–19 pandemic, but on Tristan in 1971 other aspects, such as the mortality of about 1 % in the elderly, were more like recent experience, though the frequency of chest complications was high.

It is notable that only 8 % of those affected by illness I and 12 % of those affected by illness II developed bronchospasm. The incidence of asthma was previously 45 % or 48·5 % (Woolley, 1963; Black, Thacker & Lewis, 1963) and occurred mostly after respiratory infections, while Samuels (1963) found it about 10 % with a much higher proportion of chronic sufferers who went about wheezing without requesting medical attention. Although this epidemic was said to be the worst 'sick' for between 10 and 20 years, and affected virtually all the islanders, some islanders who had bronchospasm said this was their first wheezing attack for years. This suggests that the incidence of bronchospasm is declining on the island. It is certain that there has been a great reduction in the incidence of parasitic infection, and improvement in living conditions generally, over the last 10 years. A longitudinal study on the present incidence of asthma on the island is in progress and may shed more light on the problem.

An epidemic of influenza affecting virtually the whole of a closed society in a

short space of time is a striking, even an awesome, phenomenon. A normally active community comes to a standstill. Agriculture and fishing cease. Few people are seen out and about. Whole houses and families are laid low, so that even in a closely knit society like Tristan it was sometimes difficult to find a fit person to care for the sick. At first there is a 'blitz' atmosphere in the face of adversity, but as morbidity continues morale falters. We must remember that influenza can still be a dangerous disease.

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Immunity to influenza in ferrets

V. Immunization with inactivated virus in adjuvant 65

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SUMMARY

Ferrets infected with influenza virus A2/Hong Kong/3/68 responded with a febrile reaction; the temperature was elevated by 1·0° C. or greater to a level of 40° C. or more. In addition, relatively high titres of virus were recovered from nasal washings taken 3 days after virus infection, serum antibody was produced, increased nasal protein was detected and nasal washings contained both HI and neutralizing antibody. Of four ferrets immunized with 400 CCA units of inactivated influenza virus A2/Aichi/2/68 in saline, only one produced detectable serum HI antibody, and none produced detectable nasal antibody. These ferrets were subsequently found to be susceptible to intranasal infection with influenza virus A2/Hong Kong/3/68. Thus, the temperature response, the titre of virus recovered from nasal washings and the serum HI antibody response found after virus infection was similar to that found after infection of non-immunized ferrets. However, the increase in protein concentration and the titre of HI and neutralizing antibody found in nasal washings after virus infection was detectably less than that found after virus infection of non-immunized ferrets.

Four ferrets were immunized with 400 CCA units of inactivated A2/Aichi/2/68 virus in adjuvant 65, and these ferrets produced relatively high titres of serum HI antibody but no detectable nasal antibody. After subsequent virus infection with influenza virus A2/Hong Kong/3/68, these ferrets showed a modified temperature response, reduced titres of virus in nasal washings compared to that found in nasal washings from non-immunized ferrets, no increase in nasal protein and no detectable nasal HI antibody. Thus, immunization with inactivated virus in adjuvant 65 resulted in a significant modification of the response of ferrets to challenge virus; however, the immunity was not complete, and appreciably less than that found after infection with live homologous virus.

INTRODUCTION

Previous studies have shown that ferrets were highly susceptible to infection with influenza virus (Smith, Andrewes & Laidlaw, 1933; Haff, Schriver & Stewart, 1966; Marois, Boudreault, Difranco & Pavizanis, 1971). Thus, ferrets inoculated with influenza virus A2/Hong Kong/3/68 responded with a sharp rise in temperature, rhinitis, and produced both nasal and serum antibody (Potter *et al.* 1972 *a*, *b*).

These animals may be used as a model for the study of immunization against influenza, since ferrets immune to infection with influenza virus failed to exhibit any of the above symptoms after intranasal infection with virulent virus (Francis & Stuart-Harris, 1938; Potter *et al.* 1972*a*).

In earlier studies, immunization of ferrets with inactivated A2/Hong Kong vaccine in saline failed to stimulate serum antibody, and the animals were completely susceptible to later infection with live virus (Potter *et al.* 1972*a, b*). In the present study, we report the results of immunization of ferrets with influenza virus A2/Aichi/2/68 in adjuvant 65 (Weibel *et al.* 1967; Stokes *et al.* 1969; Woodhour *et al.* 1969) and the effects of subsequent challenge with influenza virus A2/Hong Kong/3/68. The results are compared with the results of infection with virulent influenza virus on ferrets previously immunized with A2/Aichi/2/68 in saline and on non-immunized animals.

<div align="center">MATERIALS AND METHODS</div>

Virus and virus vaccine

A single pool of influenza virus A2/Hong Kong/3/68 (H3N2) was used for all ferret challenge experiments; the preparation and properties of this virus have been described previously (Potter *et al.* 1972*a*). Killed, monotypic influenza virus A2/Aichi/2/68 (H3N2) in saline (400 CCA units in 0·5 ml.) and the same virus concentration in adjuvant 65 were kindly supplied by Dr M. R. Hilleman (Merck Institute of Therapeutic Research, West Point, Pa.).

Experimental design

Groups of ferrets were inoculated intramuscularly with 400 CCA units of killed influenza virus A2/Aichi/2/68 in saline or in adjuvant A65. The temperatures of the animals were taken twice daily for 4 days and subsequently daily for 3 days after immunization, as described previously (Potter *et al.* 1972*a*); the temperatures were also taken from a group of control ferrets which had not been immunized. Ferret nasal washings were collected on alternate days from day 5 to 15 after immunization for protein and antibody determination. Serum specimens were collected by cardiac puncture before immunization and 32–34 days after immunization.

Thirty-five days after immunization, the ferrets were inoculated intranasally under light ether anaesthesia with $10^{6·5}$ EID 50 of influenza virus A2/Hong Kong/3/68; temperature readings and nasal washings were collected as described after immunization. Two temperature readings of $\geqslant 40·0°$ C. occurring in the period 24–72 hr. after virus infection was taken as a significant temperature, and two temperature readings of $\geqslant 1·0°$ C. rise above the mean pre-infection temperature was taken as a significant increase in temperature. In previous studies, non-immunized ferrets consistently showed both a significant temperature and a significant increase in temperature following intra-nasal infection with influenza virus A2/Hong Kong/3/68 (Potter *et al.* 1972*a*). Nasal washings were collected three days after infection for virus isolation and 5–15 days after infection for protein and antibody determinations. A third serum specimen was obtained 28 days after virus infection.

Virus isolation

Nasal washings taken 3 days after virus infection were stored at $-80°$ C. in PBS containing 1 % bovine serum albumin and antibiotics. The specimens were thawed, serial logarithmic dilutions were made in mixture '199' and 0·1 ml. volumes inoculated into eggs by the allantoic route. After incubation at $36°$ C. for 72 hr., the allantoic fluids were harvested and tested for virus haemagglutinin using 0·5 % fowl cells. The titre of virus was estimated by the method of Reed and Muench (1938). The identity of each virus isolated from ferret nasal washings was established by haemagglutination-inhibition tests using specific ferret antisera.

Nasal washings

Ferret nasal washings were collected by washing the nose with PBS, as described previously (Potter *et al.* 1972*a*). Nasal washings, taken 5–15 days after immunization and after virus infection for protein and antibody studies, were shaken with glass beads and then centrifuged at 3000 r.p.m. for 10 min. The supernatant fluids were concentrated 10-fold by dialysis against 30 % Carbowax, and stored at $-20°$ C.

Protein estimation

The protein concentration of 10-fold concentrated nasal washings was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Haemagglutination inhibition (HI) tests

Haemagglutination-inhibition (HI) tests and neutralization tests were carried out by the methods described previously (Potter *et al.* 1972*a*).

RESULTS

The response to infection with virulent influenza virus A2/Hong Kong/3/68

Four ferrets, which had not been previously immunized, were infected with $10^{6·5}$ EID 50 of influenza virus A2/Hong Kong /3/68 by the intranasal route, under ether anaesthesia; this was carried out at the same time and using the same virus preparation that was used to challenge ferrets previously immunized with influenza A2/Aichi/2/68 vaccines. This virus inoculum produced a sharp increase in temperature for all four ferrets; for all four animals both a significant temperature and a significantly increased temperature were recorded (Fig. 1 A, and Table 1). Nasal washings taken 3 days after virus infection all yielded virus; the titre of virus recovered in these specimens was $10^{5·25}$–$10^{6·25}$ EID 50/ml. After infection, all the animals were found to have high titres of serum HI antibody (Table 1).

Tests on ten-fold concentrated nasal washings taken 5–15 days after virus infection showed the presence of HI and neutralizing antibody in all the specimens taken 7 and 9 days after virus infection, and in three of the four specimens taken 11 days after infection. Antibody was not detected in nasal wash specimens taken five days after infection, or in specimens collected 13 and 15 days after virus infection (Fig. 2 A). The concentration of protein in nasal washings increased to a

7-2

Table 1. *Response of ferrets to infection with influenza virus A2/Hong Kong/3/68*

Ferret no.	Temperature		Virus isolation (titre)†	Change in serum HI titre	Change in nasal antibody*	
	$\geqslant 40\cdot0$	$\geqslant 1\cdot0$ rise			HI	Neut‡
174	+	+	+ (5·25)	< 5–800	< 5–20	< 2–40
175	+	+	+ (5·5)	< 5–1600	< 5–30	< 2–20
176	+	+	+ (6·25)	< 5–2400	< 5–40	< 2–60
177	+	+	+ (5·75)	< 5–800	< 5–40	< 2–40

* Change from pre-infection titre to peak, post-infection titre.
† $\mathrm{Log}_{10}\mathrm{EID}_{50}$/ml.
‡ Neutralization test.
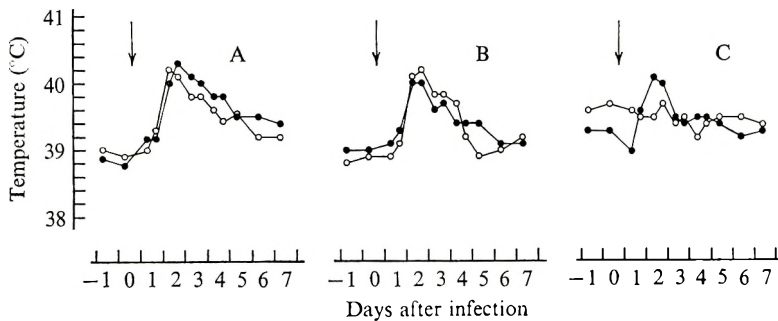
Fig. 1. Temperature response of ferrets to infection with influenza virus A2/Hong Kong/3/68. (A) Normal ferrets No. 174 and 177 (see Table 1). (B) Ferrets No. 162 and 171 (see Table 2) following immunization with 400 CCA of inactivated A2/Aichi/2/68 virus vaccine in saline. (C) Ferrets No. 164 and 166 (see Table 3) following immunization with 400 CCA of inactivated A2/Aichi/2/68 virus vaccine in adjuvant 65. ↓ — Time of virus inoculation.
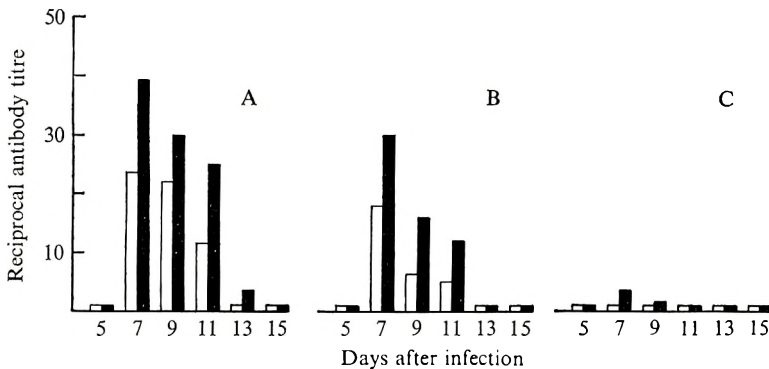
Fig. 2. Antibody titres in ferret nasal washings. Arithmetic mean titres of HI antibody (□) and neutralizing antibody (■) in × 10 concentrated ferret nasal washings collected 5–15 days after intranasal infection with influenza virus A2/Hong Kong/3/68. (A) Normal ferrets. (B) Ferrets previously immunized with 400 CCA of inactivated A2/Aichi/2/68 virus in saline. (C) Ferrets previously immunized with 400 CCA of inactivated A2/Aichi/2/68 virus in adjuvant 65.
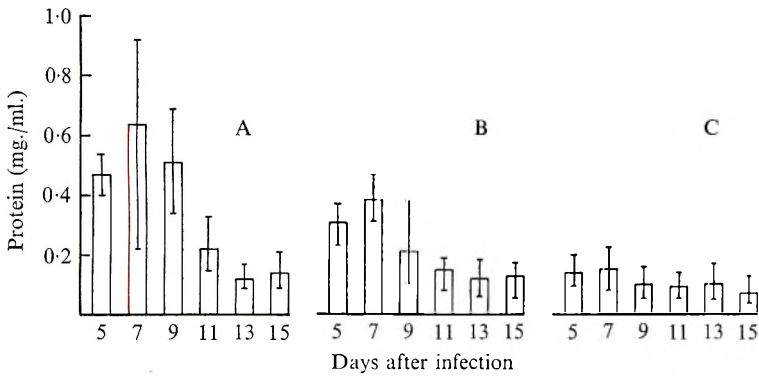
Fig. 3. Protein concentration in ferret nasal washings. Protein concentration of × 10 concentrated ferret nasal washings collected 5–15 days after intranasal infection with influenza virus A2/Hong Kong/3/68. (A) Normal ferrets. (B) Ferrets previously immunized with 400 CCA of inactivated A2/Aichi/2/68 virus vaccine in saline. (C) Ferrets previously immunized with 400 CCA of inactivated A2/Aichi/2/68 virus vaccine in adjuvant 65. □ mean protein concentration. I range of protein concentrations.

peak level at 7–9 days after virus infection; the level reached for individual animals was 3- to 5-fold greater than that found in pre-infection specimens (Fig. 3A). The protein concentration had fallen in specimens taken 9 days after infection, and the level for specimens collected 13–15 days post-infection was similar to that measured for uninfected ferrets.

Response to immunization with killed A2/Aichi/2/68 vaccine

Response to immunization

Four ferrets were each immunized with 400 CCA units of killed influenza virus A2/Aichi/2/68 in an 0·5 ml. volume by the intramuscular route, and this inoculation caused a febrile reaction. Thus, in the period 24–72 hr. following immunization, all four ferrets had a measurable increase in temperature which subsequently fell to pre-inoculation levels; however, in only one case did the temperature exceed 40° C. on two occasions, and in only one ferret did the temperature increase by 1·0° C. above the pre-inoculation level (Table 2). Immunization with the A2/Aichi/2/68 in saline did not result in the production of detectable levels of HI or neutralizing antibody in the ferret nasal washings (Table 2), and the protein concentration of these specimens remained unchanged. The vaccine did not induce detectable levels of serum HI antibody to A2/Hong Kong virus in three of the four ferrets; serum HI antibody was detected at a relatively low titre in the remaining ferret (Table 2).

Response to infection

Thirty-five days after immunization with killed saline A2/Aichi/2/68 vaccine, the four ferrets were inoculated with influenza virus A2/Hong Kong/3/68. For all four animals, this infection was followed by a sharp temperature response (Fig. 1B and Table 2). Thus, all the animals showed both a significant temperature and a significant increased temperature in the period 24–72 hr. after virus infection.

Table 2. *Response of ferrets to immunization with A2/Aichi/2/68 vaccine, and subsequent infection with influenza virus A2/Hong Kong/3/68*

Ferret no.	Response to immunization					Response to infection				
	Temperature		Change in serum HI titre	Change in nasal antibody*		Temperature		Virus isolation (titre)‡	Serum HI titre	Change in nasal antibody*
	≥40·0	≥1·0 rise		HI	Neut†	≥40·0	≥1·0 rise			HI	Neut†
162	−	+	<5–<5	<5–<5	<2–<2	+	+	+(5·25)	800	<5–10	<2–30
170	−	−	<5–<5	<5–<5	<2–<2	+	+	+(5·75)	600	<5–40	<2–60
171	−	−	<5–<5	<5–<5	<2–<2	+	+	+(5·5)	300	<5–20	<2–20
172	+	−	<5–15	<5–<5	<2–<2	+	+	+(5·5)	1200	<5–<5	<2–10

* Change from pre-infection titre to peak, post-infection titre.
† Neutralization test.
‡ $Log_{10}EID_{50}$/ml.

Table 3. *Response of ferrets to immunization with A2/Aichi/68 vaccine in adjuvant 65, and subsequent infection with influenza virus A2/Hong Kong/3/68*

Ferret no.	Response to immunization					Response to infection				
	Temperature		Change in serum HI titre	Change in nasal antibody*		Temperature		Virus isolation (titre)‡	Serum HI titre	Change in nasal antibody*
	≥40·0	≥1·0 rise		HI	Neut†	≥40·0	≥1·0 rise			HI	Neut†
163	−	−	<5–800	<5–<5	<2–<2	+	+	+(2·75)	1600	<5–<5	<2–2
164	−	−	<5–1200	<5–<5	<2–<2	+	−	−	1600	<5–<5	<2–5
165	+	−	<5–400	<5–<5	<2–<2	+	−	+(3·25)	600	<5–<5	<2–5
166	+	−	<5–1200	<5–<5	<2–<2	−	−	+(<1·5)	1200	<5–<5	<2–15

* Change from pre-infection titre to peak, post-infection titre.
† Neutralization test.
‡ $Log_{10}EID_{50}$/ml.

Virus was recovered from all four ferrets from nasal washings collected 3 days after virus infection; the titres of virus in these specimens were $10^{5 \cdot 25}$–$10^{5 \cdot 75}$ EID 50/ml. (Table 2). Serum specimens taken 21 days after virus infection from all four ferrets showed relatively high titres of HI antibody to A2/Hong Kong virus (Table 2).

The concentration of protein in nasal washings taken 5–15 days after virus infection showed that the levels had increased to a maximum at 7 days after infection; the maximum concentration of protein for individual ferrets at this time was 2- to 3-fold greater than that found in non-immunized ferrets (Fig. 3B). The protein levels in specimens taken 11–15 days after infection were similar to pre-infection concentrations (Fig. 3B). Nasal washings taken 7 days after infection from three of the ferrets contained HI antibody, and in two of these ferrets HI antibody was also present in nasal washings taken on days 9 and 11 after infection (Fig. 2B). The remaining ferret was the animal which produced a low titre of serum HI antibody after immunization (Table 2), and nasal washings taken after challenge from this animal did not contain detectable HI antibody; however, nasal washings collected on day 7 and 9 after infection both contained low titres of neutralizing antibody.

The response to immunization with killed A2/Aichi/2/68 in adjuvant A65

Response to immunization

Four ferrets were each immunized intramuscularly with 0·5 ml. of A2/Aichi/2/68 virus vaccine (400 CCA units in adjuvant 65); this procedure resulted in a measurable febrile response in three of the animals. Thus, a significant temperature was recorded in two of the ferrets in the period 24–72 hr. after immunization, but none of the animals showed a significant increased temperature (Table 3). In contrast to the result with saline vaccine, immunization with killed A2/Aichi/2/68 virus in adjuvant 65 induced serum HI antibody, and serum specimens taken 32–34 days after immunization contained HI antibody titres of 1/400–1/1200 (Table 3). However, immunization did not result in the production of detectable HI or neutralizing antibody in nasal washings (Table 3). The protein concentration of nasal washings taken 5–15 days after immunization did not change significantly from pre-immunization values.

Response to infection

Thirty-five days after immunization with A2/Aichi/2/68 vaccine in adjuvant 65, the four ferrets were infected intranasally with $10^{6 \cdot 5}$ EID 50 of influenza virus A2/Hong Kong/3/68. One ferret responded with both a significant temperature and a significant rise in temperature in the period 24–72 hr. after infection; however, the temperature response of the other three animals was modified (Fig. 1C, Table 3). Thus, for two ferrets a significant temperature was recorded but not a significant rise in temperature, and for the remaining animal the temperature remained normal (Table 3). Virus was recovered from nasal washings taken three days after infection from three of the four ferrets; the titres of virus recovered were $< 10^{1 \cdot 5}$–$10^{3 \cdot 25}$ EID 50/ml., and virus was not recovered from the remaining animal (Table 3). Thus, the titre of virus present in the nasal washings was

significantly lower than that found in unimmunized ferrets or in ferrets previously immunized with saline vaccine (Tables 1 and 2).

Infection with virulent influenza virus A2/Hong Kong/3/68 did not result in a significant increase in serum HI antibody for ferrets previously immunized with the vaccine in adjuvant 65; for one ferret the titre increased two-fold, and for the other animals the titres were similar to those before infection. Tests on nasal washings taken 5–15 days after virus infection showed no significant increase in protein above the pre-infection values (Fig. 3C) and no detectable HI antibody (Fig. 2C). However, relatively low titres of neutralizing antibody were found in nasal washings taken 7 days after virus infection from all four ferrets and, for one ferret, in the nasal wash taken nine days after virus infection (Table 3 and Fig. 2C). None of the specimens taken 5 days, or 11 or more days after virus infection contained detectable neutralizing antibody (Fig. 2C).

DISCUSSION

Previous studies have described the reaction of ferrets to infection with virulent influenza virus. The animals respond with both a significant temperature and a significant rise in temperature in the period 24–72 hr. after virus infection (Haff et al. 1966; Potter et al. 1972a). In addition, relatively high titres of virus were recovered in nasal washings of infected ferrets; there was a 3- to 5-fold increase in the concentration of protein in nasal washings; both neutralizing and HI antibody were detected in the ferret nasal washings; and the animals showed a significant rise in serum antibody (Potter et al. 1972 a, b). Since immune ferrets do not exhibit these changes after infection (Potter et al. 1972a, b), the measurement of these changes can be used as a model system to assess immunity to, and immunizing methods against, influenza virus infection.

The influenza vaccine used in the present study produced a measurable febrile response in ferrets; since this was observed after immunization with both saline vaccine and vaccine in adjuvant 65, this was attributable to the virus preparation, and not to adjuvant 65.

The temperature response to infection with virulent influenza virus A2/Hong Kong/3/68 in ferrets which had been previously inoculated with 400 CCA units of killed A2/Aichi/2/68 virus in saline was similar to that observed in non-immunized animals. Thus, immunization did not reduce the temperature response, and by this criterion immunization with this vaccine conferred no immunity to the challenge virus. In addition, the titre present in nasal washings taken 3 days after challenge was similar in both control and immunized ferrets. Three of the ferrets previously immunized with saline vaccine produced HI antibody in nasal washings after virus infection; the ferret which failed to produce nasal HI antibody was the animal which possessed a low titre of serum antibody before challenge. The concentration of protein and the titre of antibody present in the nasal washings after virus infection were measurably lower in ferrets immunized with saline vaccine than in control animals. Thus, although by two of five criteria the response of immunized ferrets to challenge was measurably less than that of control ferrets, the killed

saline vaccine did not induce significant immunity to virus challenge. These findings were similar to the results obtained with a previous saline inactivated influenza virus vaccine (Potter *et al.* 1972 *a, b*).

Immunization of ferrets with 400 CCA units of influenza virus A2/Aichi/2/68 in adjuvant 65 resulted in relatively high titres of serum antibody, but no detectable nasal wash antibody. In this respect, the ferret findings were distinct from those obtained in man where immunization with inactivated influenza virus in adjuvant 65 has been reported to induce high titres of both serum and nasal antibody (Hilleman *et al.* 1972). After immunization, the ferrets gave a modified response to challenge with influenza virus A2/Hong Kong/3/68. The animals showed a measurably modified temperature response to virus infection and nasal washings contained reduced titres of virus. In addition, the titre of serum HI antibody did not significantly increase after infection with the challenge virus. Nasal washings taken 5–15 days after virus infection from all four ferrets showed relatively low titres of neutralizing antibody, but did not contain detectable HI antibody or increased protein concentration. Thus the ferrets, though not completely resistant to the challenge virus, showed a significantly modified response to influenza virus infection. The degree of immunity to challenge with influenza virus which was conferred by prior immunization with killed A2/Aichi/2/68 vaccine in adjuvant 65 was equivalent to that observed after immunization with killed A2/Hong Kong/3/68 vaccine in Freund's complete adjuvant (Potter *et al.* 1972*b*). Immunization with killed virus in either adjuvant A65 or in Freund's complete adjuvant induced titres of serum antibody similar to that observed after infection with live virus; however, the immunity which followed natural infection was complete, whilst that which followed immunization with killed virus in either adjuvant was not complete. This failure of killed virus vaccines to induce a solid immunity comparable to that found after infection with live virus may be due to the failure of the vaccines to elicit a local antibody response.

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The post-mortem diagnosis of influenzal infection by fluorescent IgG, IgA and IgM antibody studies on necropsy blood

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SUMMARY

Necropsy blood from cases diagnosed as dying from influenza A was examined for specific antibody in the IgG, IgA and IgM fractions and a specific diagnosis of recent infection was made if either IgM or IgA antibody and low titres of IgG antibody were found. By these criteria a diagnostic rate of 77 % was found in those cases from whom no virus was isolated. The use of infected cell monolayers grown on polytetrafluoroethylene-coated slides gave a simple method of carrying out these antibody assays, and the use of necropsy blood did not require any special methods of transport of specimens to the virus laboratory.

INTRODUCTION

The virus diagnosis of influenza at necropsy is usually performed on material from the respiratory tract by either the isolation of virus or the identification of virus antigen in it by the fluorescent technique (Hers, van der Kuip & Masurel, 1968; McQuillin, Gardner & McGuckin, 1970). Both methods depend for success on the time of death after infection and on the correct collection and handling of specimens. As the humoral antibody response to influenza in the specific IgG, A and M fractions usually starts 4–7 days after the onset of symptoms, blood taken at necropsy was examined for specific antibodies to influenza virus in order to see if a diagnosis of recent infection was possible *post mortem*.

METHODS AND MATERIALS

Blood taken at necropsy from three groups of patients was studied. The first group consisted of patients from whom influenza A (H3N2) strains were isolated from the respiratory tract; the second of patients with respiratory disease thought to be due to influenza but from whom no virus was isolated; and the third control

group consisted of those dying from a variety of causes when influenza A was not occurring. Those in the first two groups died during the epidemic of influenza A which ran in Manchester from December 1971 to February 1972, and the controls died during May, June and July 1972 when no influenza A infection was detected. The ages of these patients varied from 55 to 90 in the influenzal groups and from 55 to 85 in the control group. Seven of the 13 patients in group 1, 4 of 13 in group 2, and 17 of the 25 in the control group were males. The patients in groups 1 and 2 died in Salford or Shrewsbury. The control patients in group 3 died in Salford or in Withington Hospital, Manchester.

Virus isolations

Virus isolations were made from either tracheal swabs or from extracts of lung by inoculation of rhesus monkey kidney cell cultures and the virus was identified by neutralizing haemadsorption with specific antiserum.

Fluorescent antibody studies

Monolayers of rhesus monkey kidney cells infected with influenza A (H3N2) (A2/Eng/29/70) and VERO monkey kidney cells infected with influenza B (B/Eng/32/71) viruses were used for the detection of fluorescent antibodies by the indirect technique. Darkground microscope slides, which had been thoroughly soaked and washed in haemosol, rinsed three times with demineralized water and twice with distilled water, were stored in methylated spirits until required for PTFE* coating (Goldman, 1968). Twelve or 18 clear disks were left on each slide by screening them with either drops of glycerine or the heads of No. 8 or No. 6 'Posidrive' wood screws during spraying. These PTFE slides were placed in 100 mm. square plastic Petri dishes (Sterilin Limited) and 'sterilized' in a hot-air oven at 60° C. overnight. Each petri dish contained five slides (76 mm. × 25 mm.), two of which had been slightly reduced in width by removing a small sliver of glass so that they fitted snugly on the petri dish bottom. Drops of cell suspension containing 300,000 cells per ml. were placed on each of the disks and the cells allowed to grow out for 24 hr. at 37° C. in plastic or glass candle boxes sealed with electrical tape. Plastic boxes were protected from the candle flame by a sheet of aluminium foil. The cell culture growth medium was Parker '199' with 10 % calf serum, 2 % of 4·4 % sodium bicarbonate solution and the usual antibiotics. After 24 hr. the growth medium from each disk was removed with a pasteur pipette and replaced with a drop of maintenance medium (in which 1 % embryo calf serum was substituted for 10 % calf serum) containing from 10^5 to 10^6 influenza virus cell culture infectious doses per ml. The dose of virus chosen showed fluorescent material in approximately 10 % of culture cells after 24 hr. incubation. At this time the slides were removed from the Petri dishes and washed twice in buffered saline, twice in acetone and then fixed in acetone for 3–5 min. and air dried for 20 min. The slides were stored at − 30° C. until required. As slides kept for at least 3 months, batches of 40 to 50 were made at one time. Sera to be tested were inactivated at 56° C. for

* Polytetrafluoroethylene spray with bonding additive obtainable from Fisons Scientific Apparatus, Loughborough or coated slides (6 mm. diameter disks) from C. A. Hendley & Co., Victoria Road, Buckhurst Hill, Essex.

Table 1. *Influenza A (H3N2) fluorescent antibody titres in necropsy sera of patients probably dead of influenza (Dec. 1971–Feb. 1972)*

		Group 1 (virus isolated)					Group 2 (virus not isolated)		
		Titre in serum fractions					Titre in serum fractions		
Case no.	Age and sex	IgG	IgM	IgA	Case no.	Age and sex	IgG	IgM	IgA
1	68 M	18	< 6	6	14	82 M	6	6	6
2	77 M	12	< 6	6	15	59 F	12	< 6	6
3	67 F	≥ 486	54	324	16	74 F	≥ 486	6	486
4	59 M	18	< 6	< 6	17*	88 F	162	< 6	6*
5	62 M	≥ 486	12	108	18	80 F	18	< 6	6
6	55 M	18	< 6	< 6	19	79 M	24	< 6	6
7	67 F	24	< 6	< 6	20	72 M	18	< 6	6
8	73 F	18	< 6	< 6	21	87 F	≥ 486	54	54
9	86 M	80	< 5	20	22	90 F	18	6	6
10	64 F	640	80	40	23	83 F	6	< 6	6
11	54 F	45	< 5	< 5	24	80 F	12	< 6	< 6
12	55 M	30	< 5	< 5	25	67 F	5	< 5	< 5
13	75 F	≥ 405	45	45	26	59 M	1280	160	80

* Recently vaccinated.

half an hour and either screened or titrated using two- or three-fold steps, starting at an initial dilution of either 1/5 or 1/6. Each serum dilution was placed on one disk, this having first of all been demarcated from its neighbour by means of a Shachihata Artline 70 marking pencil. This ensured that one serum sample did not run into its neighbour. The serum dilutions were left on the infected monolayers for one hour at 37° C. These were washed for 5–10 min. in three changes of buffered saline and stained for 30 min. with specific antigammaglobulin fluorescent conjugates obtained from either Behringwerke (anti-IgG), Nordic (anti-IgM), or Wellcome Reagents Limited (anti-IgG, A & M). The dilutions of the conjugates used were determined by previous titrations. The preparations were then examined with a Reichert Zetopan microscope fitted with quartz iodine illumination (Cradock–Watson, Bourne & Vandervelde, 1972).

RESULTS

The individual antibody titres of influenza A virus (H3N2) in the IgG, IgM and IgA globulin fractions for the two groups of respiratory disease patients are given in Table 1. The numbers with different titres to IgG, IgM and IgA in the control group are given in Table 2 and a summary of the findings with the number of serological diagnoses of recent influenza from these single specimens of necropsy blood is given in Table 3.

Seven sera from the 13 patients in whom influenza A virus (H3N2) was found had either IgA and/or IgM antibody present, or IgA and titres of IgG of less than 1/100, the latter indicating that the IgG titres were rising and had not yet reached those associated with convalescence. In those cases not yielding virus, 10 of the

Table 2. *Influenza A (H3N2) virus fluorescent antibody titres*
in 25 control necropsy sera (May to July 1972)

Antibody titre	No. with given titre of		
	IgG	IgM	IgA
< 5	3	25	24
5	0	0	1
15	8	0	0
45	11	0	0
135	3	0	0

Table 3. *Fluorescent antibodies to influenza A (H3N2) virus*
in necropsy sera

	Virus isolated	No. tested	No. with IgG	No. with IgM	No. with IgA and low IgG (< 100)	No. with IgA and IgG (> 100)	Serologically indicative of recent influenza (%)
Possible clinical influenza Dec. 1971 to Feb. 1972	Yes	13	13	4	3	0	54
	No	13	13	5	5	1*	77
Controls June–July 1972	Not tested	25	22	0	0	1	0

* Recently vaccinated.

13 sera had these findings in contrast to the control group in whose sera IgM antibody was absent and only one had IgA antibody present. The presence of IgA in this case was associated with an IgG level of more than 1/100. In group 2, in which no virus was isolated, one patient who had been immunized had IgA but no IgM, and an IgG antibody titre of 1/162. As these titres could have been a sequence of vaccination and not natural influenzal infection no retrospective diagnosis could be made.

Five of the seven patients with high levels of IgG, IgM and IgA antibodies had given a definite history of influenza or respiratory disease for a week or more, while the remaining two were stated to have had acute lower respiratory symptoms for only 24 hr. before death. All those with low antibody titres had a history of respiratory disease of less than a week, except one woman with leukaemia – a disease which might have affected her antibody response.

Serum end-points were taken as the highest dilution which gave obvious fluorescence. Dilutions giving fluorescence which was probably specific but of poor intensity were ignored. This latter degree of fluorescence was not seen in any of the control sera, but was present in two of the sera from patients yielding virus and in two of those who were virus-negative.

All but two of the sera from the respiratory deaths had antibodies to influenza B in the IgG fraction, but none had any in the IgA or IgM fractions. In the control group one man had IgA and IgM antibodies to influenza B, indicating recent infection. This man lived in an area where one of the only two influenza B viruses isolated this summer in Manchester was found. He had a long history of heart disease but suffered from a 2-week period of general malaise finishing 2 weeks before his death. This was due to multiple thrombi associated with heart disease, and the myocardium contained numerous areas of healing degenerative foci highly suggestive of a recent myocarditis.

DISCUSSION

The use of necropsy blood for the post-mortem diagnosis of influenzal infection appears to be a helpful adjunct to virus isolation, which in our hands was only successful in half our suspected cases. Using the presence of IgM or IgA with low levels of IgG as the criteria of recent influenzal infection, 77 % of cases suspected of influenza, but from whom no virus was isolated, could be diagnosed. Even if the history of illness was apparently only 24 hr. a diagnosis was sometimes possible. By combined virus isolation and antibody determination, diagnosis was achieved in 88 % of our respiratory cases, presuming that all 26 were in fact due to influenza.

Necropsy blood is easily obtained and can be sent to the virus laboratory without any of the special precautions necessary for materials used for virus isolation. Immediate necropsy is not necessary as the efficiency of the fluorescent antibody technique is not affected by possible delay in receiving the blood at the laboratory. The method of antibody assay used is simple, as up to 18 serum dilutions can be tested on a single slide, and these can be prepared in batches and kept frozen until required.

Cell cultures on PTFE-coated slides have also been satisfactorily used for the estimation of antibodies in the specific immunoglobulin fractions with mumps, measles, herpes simplex virus, cytomegalovirus, adenoviruses and some enteroviruses. With monkey kidney cell suspensions the monolayers will readily grow out on the disks in individual drops, but with continuous cell lines or with fibroblast cells it is preferable to flood the slides with growth medium after the cells have settled on the glass, otherwise some of the cell monolayers may not spread out, the size of the individual drops being very critical with these cells. In practice those viruses which need more than 24 hr. to produce a fluorescent effect are mixed with the cell suspension at the time of seeding the slides, and subsequent inoculation of prepared monolayers with virus is not necessary.

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An epidemiological study of *Pseudomonas aeruginosa* in cattle and other animals by pyocine typing

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SUMMARY

The high incidence (80·1 %) of *Pseudomonas aeruginosa* pyocine type 1 in bovine mastitis in Israel suggested some degree of selectivity under local conditions. Contrary to other reports, cattle and calves showed a high rate of faecal carriage of this organism. The water supply on farms was often contaminated. The presence of certain pyocine types in the udder, gut or water occasionally led to their transmission from one reservoir to another; however, many types did not seem to spread.

P. aeruginosa was found in association with infections in various animals and was present in many locations such as a mouse breeding house and a chick hatchery.

INTRODUCTION

Pseudomonas aeruginosa is considered to be a natural inhabitant of the human intestine and common enough in water and soil (Editorial, 1969). In recent years the significance of this organism as a pathogen of man (Editorial, 1966) became well established.

A perusal of literature indicated the need for additional information on the association of *P. aeruginosa* with animals. The organism is not considered to be a normal inhabitant of the intestinal tract of most animals, however, various infections were occasionally reported (Hoadley & McCoy, 1968). Attention was given to its role in bovine mastitis, but it is not regarded as a common cause of this disease, only of concern in some herds (Schalm, Carroll & Jain, 1971).

The frequent occurrence of *P. aeruginosa* mastitis in Israel prompted a previous investigation of this problem (Ziv, Mushin & Tagg, 1971). The present study covers a broader field and is concerned with the following topics: (1) the epidemiology of *P. aeruginosa* mastitis in cattle and the faecal carrier rate in cattle and calves, (2) the association of this organism with various animals, and (3) its occurrence in water on farms.

The application of pyocine typing of isolates from various sources allowed the labelling of strains and the assessment of their relationship.

MATERIALS AND METHODS

Isolation of Pseudomonas aeruginosa

P. aeruginosa isolations from udders, rectum and faecal matter from cattle and from water supplies were carried out in our laboratory. Additional cultures from a

variety of animals, often from pathological conditions, came on nutrient agar or
Kligler iron agar.

Milk samples were obtained from two sources, from bovine udders from farms
and from a slaughterhouse. The samples were taken with aseptic precautions,
incubated at 37° C. for 24 hr. and plated out on sheep blood agar.

In the examination of material of intestinal origin, preliminary tests indicated
the advisability of using large samples. Thus heavily charged rectal swabs or large
faecal samples were placed in 5 ml. of 0·03 % cetrimide liquid medium (Brown &
Lowbury, 1965), and incubated at 37° C. for 24 hr. A large loopful of each suspen-
sion was subsequently transferred to another tube of cetrimide medium for 48 hr.
incubation. Water samples were placed in cetrimide medium in the approximate
proportion of 1:1. At a later stage of this study, the second series of cetrimide
cultures were incubated in a water bath at 42° C. Plating was done from cetrimide
tubes on cetrimide agar or SS (Difco) agar.

Identification of Pseudomonas aeruginosa

Preliminary identification of *P. aeruginosa* from blood agar plates was on colony
form, type of haemolysis and odour. These colonies and colonies from cetrimide
and SS agar were further examined on the basis of biochemical reactions in Kligler
iron agar, positive oxidase test and production of chloroform-soluble pyocyanin, or
occasionally pyorubin, in the liquid medium A of King, Ward & Raney (1954),
which was incubated at 32° C. up to 7 days. These characteristics were sufficient
for the identification of *P. aeruginosa*, while non-pigmented strains were further
tested for growth in broth at 42° C. on two successive transfers with a straight
needle, and on the production of gaseous nitrogen in Durham tubes in nitrate
broth. Pyocine production was another useful diagnostic feature.

Pyocine typing

The procedure for pyocine typing followed with some modifications the scheme
of Gillies & Govan (1966) and Govan & Gillies (1969), which lists 37 pyocine types.
Some isolates, not classified by these workers, were allotted designations UC_1 to
UC_9 (Tagg & Mushin, 1971). The major modification in the technique was the
introduction of an apparatus ('broomette') for the simultaneous streaking of
indicator strains (Tagg & Mushin, 1971). Besides the original eight indicator
strains I_1 to I_8 of Gillies & Govan (1966), five additional indicators A to E of
Govan & Gillies (1969) for the subdivision of pyocine types 1 and 10 into subtypes
a to h were used. The introduction of two of our own indicator strains, labelled
I_A and I_B (Tagg & Mushin, 1971) allowed for an additional division of pyocine
types into subtypes labelled + or −, on the basis of the activity of a potential
producer on the above two indicators. Accordingly, strains were recorded as $1^{+-}a$,
$10^{--}h$, 3^{++}, and similarly.

Each strain was typed at least twice, using composite growth picked up by a
loop sweep from a 24 hr. agar plate. Colonies showing a distinct dissociation were
on occasions individually typed. In the tabulation of the distribution of pyocine
types, the isolation of two or more types from a single specimen was recorded,
while identical types were only once listed.

Table 1. *Distribution of pyocine types in* Pseudomonas aeruginosa *strains isolated from bovine mastitis on farms and from bovine udders in a slaughterhouse*

Pyocine	116 strains from farms		50 strains from slaughterhouse	
Type and subtype	No.	%	No.	%
1^{++}a	7	6·0 ⎫	1	2·0 ⎫
1^{++}b, 1^{+-}b	17	14·7 ⎪	13	26·0 ⎪
1^{++}c, 1^{+-}c, 1^{--}c	9	7·8 ⎪	10	20·0 ⎪
1^{+-}d	2	1·7 ⎬ 80·1	0	0 ⎬ 72·0
1^{+-}f, 1^{--}f	10	8·6 ⎪	0	0 ⎪
1^{++}h, 1^{+-}h, 1^{--}h	45	38·7 ⎪	9	18·0 ⎪
1^{+-}uc, 1^{--}uc	3	2·6 ⎭	3	6·0 ⎭
10^{++}b	0	0 ⎫	2	4·0 ⎫
10^{+-}c	1	0·9 ⎬ 3·5	1	2·0 ⎬ 10·0
10^{++}h	3	2·6 ⎭	2	4·0 ⎭
3^{++}, 3^{+-}	7	6·0 ⎫ 9·4	3	6·0 ⎫ 16·0
Other types	4*	3·4 ⎭	5†	10·0 ⎭
Unclassifiable	6‡	5·2	0	0
Untypable	2	1·7	1	2·0

uc = unclassifiable subtypes.
* = types: 2^{--}, 5^{++}, 13^{--}, 35^{--}, one of each type.
† = types: 2^{--}, two; 4^{+-}, 6^{++}, 30^{--}, one of each type.
‡ = three were UC$_2$.

RESULTS

Pyocine types from bovine mastitis on farms

A total of 116 *P. aeruginosa* strains were isolated from cows' udders, some of which were re-examined on a few occasions, within a time interval of at least 2 months. As shown in Table 1, pyocine type 1 represented by 80·1 % isolations was predominant, and the most frequently encountered subtype was 1h, pattern 1^{++}h being the most common. The remaining 23 strains belonged to six pyocine types, or to the unclassifiable or untypable group.

Pyocine types from bovine udders from a slaughterhouse

Of a total of 50 *P. aeruginosa* strains (Table 1) isolated from udders in a slaughterhouse, 72·0 % were pyocine type 1, with a scatter of subtypes. Type 10 was responsible for 10 % isolations, while the remaining isolates were represented by five pyocine types and one untypable strain.

Pseudomonas aeruginosa *in faeces of cattle, calves and in water*

The survey of animal faecal carriers included cattle and calves from nine farms and a slaughterhouse. Contrary to reports found in the literature and to be discussed later, the faecal carrier rate was high (Table 2), reaching 60·0 % in calves and 32·2 % in cattle. It fluctuated in being lower in calves on farms and much higher in calves in a slaughterhouse, as compared with cattle in these locations.

A perusal of data from individual farms also indicated fluctuation in the faecal carrier rate. *P. aeruginosa* was not recovered from faecal specimens from groups

Table 2. *Incidence of* Pseudomonas aeruginosa *in bovine faecal carriers and in water samples*

Location	Source	Total no. examined	Positive No.	Positive %
Nine farms	Cattle	185	45	24·3
	Calves	33	6	18·2
Six farms	Water	16	9	56·3
Slaughterhouse	Cattle	59	19	32·2
	Calves	35	21	60·0
	Water	2	1	

Table 3. *Incidence of* Pseudomonas aeruginosa *faecal carriers in groups of calves of various ages*

Calves	$<1\frac{1}{2}$	3–5*	4*	>4*	6–9 (a)	6–9 (b)	12 (c)	12 (d)
No. examined	13	11	5	19	5	5	6	4
No. positive	2	3	3	15	0	0	0	4
% positive	15·4	27·3	60·0	78·9	0	0	0	100

The column group heading is "Age in months".

* From slaughterhouse, other calves from farms. Figures in parentheses refer to groups of calves from different farms.

of twenty and twelve cows on two farms with cases of subclinical mastitis. On the other hand, on a farm where only recently a few cases of *Pseudomonas* mastitis were diagnosed, the faecal carrier rate in cows ranged from 10 % to 50 % and for calves up to 6 weeks of age it was 15 %.

It is of interest to note the high rate of isolation of *P. aeruginosa* from water. Of sixteen samples of water from six farms, 56·3 % were positive, and each farm was represented. On two farms, where bovine faecal carriers were not detected, one out of two, and two out of three water samples were positive for this organism.

Faecal carrier rate of Pseudomonas aeruginosa *in groups of calves of various ages*

Table 3 presents the results of examination of faecal matter from calves under 6 weeks till 1 year of age. In a slaughterhouse the faecal carrier rate was as high as 78·9 % for calves over 4 months old. On farms, calves were faecal carriers when under 6 weeks and when 1 year old. The negative specimens came from three groups of calves, between 6 and 9 months, and under 12 months of age.

Pyocine types from bovine faecal carriers and from water on farms and in a slaughterhouse

In Tables 4 and 5, the figures referring to pyocine types of *P. aeruginosa* strains from bovine faecal carriers represent single and multiple isolates and therefore are higher than the figures which indicate the incidence of the species in Tables 2 and 3.

The survey of pyocine types in faecal carriers on farms (Table 4) showed that the occurrence of type 1 (36·2 %) was much lower than in udders (80·1 %). In all,

Table 4. *Distribution of pyocine types of* Pseudomonas aeruginosa *strains isolated from bovine faecal carriers and from water on farms*

Pyocine Type and subtype	58 strains from cattle No.	%	10 strains from calves No.	%	9 strains from water No.	%
1++a	1	1·7	0		1	
1++b, 1+-b	5	8·6	1		0	
1+-c	2	3·4	0		0	
1++f, 1--f	3	5·2 } 36·2	0 } 30·0		0 } 44·4	
1+-g	1	1·7	0		1	
1++h, 1--h	2	3·4	0		1	
1+-uc	7*	12·0	2		1	
10++c, 10++d	0	0 } 1·7	0 } 0		2 } 22·2	
10++h	1	1·7	0		0	
3++	12	20·7	3		0	
2--	5	8·6 } 50·0	0 } 4		0 } 0	
Other types	12†	20·7	1‡		0	
Unclassifiable	2	3·4	0	0	0	0
Untypable	5	8·6	3	30·0	3	33·3

Figures referring to strains from faecal carriers represent single and multiple isolates.
* Five subtypes had pattern + − − + − .
† Types: 5+-, 27++, 29--, 35--, two of each type; 8--, 11--, 31--, 37+-, one of each type.
‡ Type: 35--.
Unclassifiable types were UC$_3$.

Table 5. *Distribution of pyocine types of* Pseudomonas aeruginosa *strains isolated from bovine faecal carriers and from water* in a slaughterhouse*

Pyocine Type and subtype	22 strains from cows No.	%	36 strains from calves No.	%
1++b	0		2	
1++c, 1+-c	5		3	
1+-d	1		0	
1--f	0 } 63·6		2 } 36·1	
1++g	1		0	
1++h, 1+-h	3		5	
1+-uc, 1--uc	4		1	
10+-c	1 } 13·6		2 } 11·1	
10+-d, 10+-f	2		2	
3++	3	13·6	15	41·7
Other types	0	0	3†	8·3
Untypable	2	9·1	1	2·8

Figures referring to strains represent single and multiple isolates from faecal carriers.
* Two pyocine types: 1++a and 36+-.
† Types: 6++, two; 12+-, one.

there was a varied collection of pyocine types, comprising twelve types, and of unclassifiable and untypable strains. Strains from calves and water showed a limited range of pyocine types, and the occurrence of type 1 was listed as 30·0 % and 44·4 % respectively. From the faecal carriers and water samples a compara-

Table 6. Pseudomonas aeruginosa *in cows, calves
and in the water on particular farms*

	Pyocine types in			
	Faeces			
Farm code	Cows	Calves	Udder	Water
B	1+⁻uc*	1+⁻uc*	—	—
	1+⁻c	1+⁻b	—	—
	1--f	3+⁻	1--f	—
	10++	UT	UC	UT
P	UC, UT	0	1++h	10++a, 10++c
S	1++a	1++a	UC	—
	1++h	—	—	—
	27++, 37+⁻	—	—	—

* The same subtype, $+--+-$. UT = untypable. UC = unclassifiable.

Table 7. Pseudomonas aeruginosa *pyocine types from pathological material from
various animals*

Animal	No.	Pyocine type
Goose	19*	UT*
Goose	2	1++b, 1+⁻b
Turkey	1	1++e
Chicken	1	1++b
Lamb	2	10++b, 3++
Cow	1	1+⁻c
Calf	8	UC(3), 1+⁻c(2), 1++b, 3++(2)
Mink	1	27++
Fox	1	UT
Otter (*Lutra* sp.)	1	3++
Skunk (*Vormela* sp.)	1	1+⁻c

* All of one type as shown with an additional indicator. Figures in parentheses are numbers
of each pyocine type. UC = unclassifiable. UT = untypable.

tively high number of untypable strains was isolated. Some of them proved to be
sensitive to phage typing which was carried out by the courtesy of Mrs R. Ziv.
Especially in dealing with atypical non-pigmented strains, a pyocine or a phage
pattern were of diagnostic significance.

Data presented in Table 5 indicate that pyocine type 1 was frequently en-
countered amongst cows in a slaughterhouse (63·6 %), while amongst calves the
figure was lower (36·1 %). In the material from calves pyocine type 3 was most
common (41·7 %). The examination of two water samples yielded two pyocine
types from one of them, namely 1++a and 36+⁻.

Pyocine types in cows, calves and water on particular farms

Table 6 gives a representative set of data on the occurrence of pyocine types in
cows and calves of the same herds, namely in the udder and the intestine, and in
water supplies. Occasionally the same pyocine type was recovered from different

Table 8. *Distribution of pyocine types in* 88 Pseudomonas aeruginosa *strains isolated from laboratory mice*

Pyocine	Strains	
Type and subtype	No.	%
1^{+-}a	1	1·1 ⎫
1^{+-}b	2	2·2 ⎪
1^{+-}c, 1^{--}c	13	14·7 ⎬ 38·6
1^{++}h	1	1·1 ⎪
1^{--}f	3	3·3 ⎪
1^{+-}uc, 1^{--}uc	14*	15·9 ⎭
5^{+-}	21	23·9 ⎫
13^{--}	7	10·0 ⎬ 35·3
Other types	3†	3·3 ⎭
Unclassifiable	12‡	13·6
Untypable	11	12·5

* Eleven subtypes had pattern: − − − + − .
† Types: 2^{--}, 11^{--}, 29^{+-}, one of each type.
‡ Two were UC$_4$ and six were UC$_7$.

sources, such as the faeces of cows and calves, from udder and faeces, or possibly from water and faeces (untypable strain). However, usually, there was a greater range of types in the faecal material.

Pyocine types from pathological material from various animals

The material was obtained at post-mortem examinations from animals with various pathological conditions, such as bacteraemia, diarrhoea or lung infection. A set of 19 cultures, isolated from a flock of geese dying of bacteraemia (Table 7), was untypable with the indicators of Gillies and Govan (1966). However, an additional indicator was sensitive to all the strains examined, thus demonstrating their similarity.

Specimens from various animals yielded *P. aeruginosa* of different pyocine types, some unclassifiable or untypable. The only strain which was not encountered in the cultures listed in previous Tables was subtype 1^{++}e from a turkey.

Pyocine types in laboratory mice

The high incidence of *P. aeruginosa* in colonies of mice in a breeding unit provided an opportunity of sampling an additional source. The mice were of inbred lines raised under SPF (specific pathogen free) conditions, however *P. aeruginosa* became an established inhabitant. The specimens were faeces and water from drinking bottles in the cages, and occasionally organs from mice obtained at post-mortem examinations.

It can be seen in Table 8 that amongst 88 strains examined, pyocine type 1 was predominant (38·6%) and type 5^{+-} was next in the frequency of appearance (23·9%). The occurrence of unclassifiable or untypable strains, 13·6 and 12·5% respectively, was comparatively high.

Table 9. *Distribution of pyocine types in* 46 Pseudomonas aeruginosa *strains isolated in a chick hatchery*

Source of specimen	Pyocine Type and subtype	No. of strains
Dead embryo	1^{+-}c	2
1-day-old chicks	1^{+-}c	2
	1^{++}b, 1^{+-}b	2
	1^{++}h	2
	3^{++}	3
	5^{+-}, 5^{--}, UC, UT	4
Eggs	1^{+-}c, 5^{--}	2
Solution before dipping eggs	1^{+-}c	4
	1^{++}h	1
Solution after dipping eggs	1^{+-}c	21
Miscellaneous	1^{+-}h, UC, UT	3

UC = unclassifiable. UT = untypable.

For experimental purposes some mice were irradiated and many died of *Pseudomonas* septicaemia. Of twenty-four strains, the majority were either 1^{+-} or untypable.

Pyocine types in chick hatchery

Another set of *P. aeruginosa* cultures came from a chick hatchery. As seen in Table 9, the predominant type was pyocine 1^{+-}c, which was found in 4 samples of a solution before the dipping and in 21 after the dipping of eggs. It was also isolated from two dead embryos and from an egg shell. The pyocine types were similar to those encountered in other locations, but few were unclassifiable or untypable.

DISCUSSION

The present study indicated a close association of *P. aeruginosa* pyocine type 1 with bovine mastitis on farms in Israel. The incidence was high, 80·1 %, while the previously reported figure by Ziv, Mushin & Tagg (1971) was 65·7 %. The introduction of subtypes a to h (Govan & Gillies, 1969) and of a subdivision into + and − subtypes (Tagg & Mushin, 1971) allowed for more exact fingerprinting of strains. In this series of strains, pattern 1^{++}h was most frequently encountered. The above data, with some deviation, were comparable with those recorded for cattle in a slaughterhouse, the incidence of type 1 being 72·0 %.

The faecal carrier rate of pyocine type 1 in adult cattle and in calves on farms was lower, being 36·2 % and 30·0 % respectively, and in a slaughterhouse 63·6 % and 36·1 % respectively. This pyocine type was found in four out of nine *P. aeruginosa* strains from water on farms. In colonies of laboratory mice type 1 was recovered from 38·6 % of samples from these animals and from water bottles in their cages. It was also isolated from clinical material from a variety of animals, but in this series of cultures there was a disproportionate weighting of a number of untypable strains from one source. In a survey of *P. aeruginosa* in a chick hatchery type 1 was predominant, especially type 1^{+-}c which was found in various locations.

It is well documented that pyocine type 1 is the most commonly encountered type in material from human sources. In Israel, specimens from two hospitals accounted for the incidence of 37 % and 45 % (unpublished data). The figure in a survey in Scotland was 34·2 % (Govan & Gillies, 1969), in Australia 31 % (Tagg & Mushin, 1971) and in clinical isolations in U.S.A. 52·1 % (Heckman, Babcock & Rose, 1972).

It seems that although the high incidence of pyocine type 1 in bovine mastitis in Israel is the reflection of its common occurrence in the environment, the exceptionally high figure indicates a certain selectivity. The prolonged presence in the cow's udder may be partly due to the chronic character of *Pseudomonas* mastitis.

Attention was given to statements found in the literature that faecal carriers of *P. aeruginosa* appeared occasionally amongst animals associated with man. In a survey by Hoadley & McCoy (1968) the percentage of positive samples for man was 11·5 %, while transient carriage (9·45 %) in calves under four weeks of age, only on some farms, and occasional carriage in some other animals in proximity to man was observed. A study by Matthews and Fitzsimmons (1964) on the incidence of *P. aeruginosa* in the intestine of calves indicated that it was exceptional for this organism to be present in these animals by the time they attained the age of 8 weeks.

Our results, contrary to other reports, indicated a high faecal carrier rate both in adult cattle and in calves. The highest carrier rates, 78·9 % and 60·0 % were encountered in a slaughterhouse in calves over the age of 4 months and at 4 months, and on farms the organism appeared in young calves under 6 weeks and at 1 year of age. The faecal carrier rate in adult cattle in the above locations was 32·2 % and 24·3 %.

As previously mentioned, *P. aeruginosa* is common enough in water and soil (Editorial, 1969). Human faeces and sewage were shown to be its normal habitat, while natural waters were found to be devoid of this organism (Ringen & Drake, 1952). A contaminated warm water system used for spray udder washing was considered to be a source of infection of cattle (Curtis, 1969). In a collection of *P. aeruginosa* strains (Csiszar & Lanyi, 1970), 218 were from water and sewage, and some originated from a municipal water supply. In our survey nine out of sixteen water samples from farms carried this organism.

Pyocine typing was found to be useful in examining the epidemiological aspects of our survey. *P. aeruginosa* types isolated from cows' udders, from faecal material and from water on particular farms were compared. Occasionally the same pyocine type appeared in udder and faeces or in water and faeces. Shooter *et al.* (1966) in their survey of faecal carriage of *P. aeruginosa* in hospital patients, observed that although numerous strains appeared in faeces, many did not seem to spread. Similarly, in our study, the high incidence and selectivity for pyocine type 1 in cows' udders and the presence of many other pyocine types in faecal material did not point to a frequent transmission from one reservoir to another. Apparently more data should be collected on the dissemination of *P. aeruginosa* under various environmental conditions.

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The direct fluorescent antibody test for detection of *Brucella abortus* in bovine abortion material

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SUMMARY

The direct fluorescent antibody test was assessed for detection of *Brucella abortus* in bovine abortion material. *Br. abortus* organisms could be readily detected as characteristic intra-cellular clumps, in smears stained with fluorescent antibody. Counterstaining with Evans Blue was necessary to suppress tissue auto-fluorescence. The method was specific and enabled brucella infection to be differentiated, *inter alia*, from Q-fever infection.

INTRODUCTION

At present the methods available for detection of *Brucella abortus* in abortion material include direct staining by the modified Köster method (Christoffersen & Ottosen, 1941) and the modified Ziehl Neelsen method, or isolation of the organism by culture and guinea-pig inoculation. When used conjointly these methods enable an accurate diagnosis to be made in most cases. Unfortunately, isolation by animal inoculation takes a long time and is not always practicable and so reliance is often placed on direct microscopical examination and culture. However, many samples of abortion material are unsuitable for cultural examination, even on selective media, because of overgrowth by contaminating organisms. In these circumstances presumptive diagnosis may depend solely upon the results of microscopical examination.

Although relatively specific for brucellas, the modified Köster's procedure will not differentiate them from *Coxiella burnetii* and in areas where Q-fever is prevalent this may cause serious problems in diagnosis. The direct fluorescent antibody test (FAT) appeared to offer a more specific alternative to the modified Köster's procedure and so was assessed for its value in detecting *Br. abortus* in bovine abortion material.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study were from stock cultures maintained at this laboratory.

Pathological material

Material from bovine abortions, which was submitted for routine diagnosis, was used in this study. Samples were examined by conventional staining, culture and

in some cases also by animal inoculation. Smears of abortion material known to be infected with *Coxiella burnetti*, *Campylobacter fetus* (*Vibrio fetus*), *Leptospira* spp., *Salmonella dublin*, *Salmonella enteritidis*, *Listeria monocytogenes* and *Aspergillus fumigatus* were also available for examination.

Preparation of antisera

Antiserum to *Br. abortus* was produced by intramuscular injection of a single dose of *ca.* 10^{10} *Br. abortus* 544 organisms into 4-month-old rabbits. Blood samples were collected at weekly intervals and those showing high titres in agglutination, complement fixation and precipitin tests at 4 weeks after inoculation, were pooled and stored frozen at $-20°$ C. until required.

Preparation of conjugate

Pooled high titre serum was fractionated by repeated precipitation with sodium sulphate (Keckwick, 1940). The γ-globulin was dialysed against PBS at 4° C. until sulphate was no longer detectable with $BaCl_2$ and, after clarification by centrifugation, the protein content determined by ultra-violet absorption spectroscopy according to Cullen & Corbel (1970). The protein concentration was adjusted to 1·0 g. per 100 ml. and the pH raised to 9·5 by addition of 1·0 M-Na_2CO_3. Fluorescein isothiocyanate (FITC) (Isomer 1; British Drug Houses, Poole) dissolved in 0·1 M carbonate-bicarbonate buffer, pH 9·5, was then added to give a final proportion of 10 mg. FITC per 1·0 g. of protein, and the mixture agitated in a sealed flask on a magnetic stirrer for *ca.* 18 hr. at 4° C. The reaction was stopped by adjusting the pH to 7·0 and excess FITC removed by dialysis against PBS at 4° C. Residual unreacted FITC and labelled globulin with non-speciс fistaining activity were removed by absorption with acetone-dried bovine liver powder at a final concentration of 50 mg. per ml. After absorption for 2–3 hr. at 4° C. the globulin was recovered after centrifugation at 30,000 *g* for 15 min. The conjugate was assayed for FITC/protein ratio according to The & Feltkamp (1970) and for free FITC by thin layer chromatography on Sephadex G200 superfine according to a modification of the method of Morris (1964).

The conjugate was adjusted to pH 7·5 and sodium azide added to 0·1 % final concentration and the preparation sterilized by membrane filtration. For storage, the material was kept frozen in 1·0 ml. ampoules at $-20°$ C.

Performance of the test

Smears of abortion material, usually bovine placenta or foetal stomach contents, were made on clean microscope slides as soon as possible after collection. The smears were fixed by thorough but not excessive heating. Sufficient conjugate was then pipetted on each slide to cover an area of *ca.* 10 mm. × 20 mm. of the smear. Reaction was allowed to proceed in a moist atmosphere at 37° C. for 30 min. Surplus conjugate was rinsed off with PBS and the smears washed in two changes of PBS with constant agitation. The preparations were then counterstained with 0·25 % Evans Blue in 0·15 M-NaCl for 1–2 min., the surplus stain rinsed off in PBS and the preparations mounted in buffered glycerol (90 %, v/v, glycerol A.R.; 10 %, v/v, 0·1 M phosphate buffer, pH 8·0).

Smears were examined with a Leitz Orthoplan fluorescent microscope fitted with HBO 200 mercury burner, 4 mm. BG 38 + 5 mm. BG 12 exciting filters and incident illuminator (after Ploem) with TK 495 dichroic beam-splitting mirror and 490 nm. suppression filter. Photographs were made on Kodak High Speed Ektachrome film (EH 135–20) with exposure times of *ca* 2 min.

Evaluation of smears

Smears were scanned for fluorescent areas under low power magnification ($\times 125$) and on locating any such areas closer inspection was made under high power magnification ($\times 1250$). For placental smears only the presence of fluorescent intracellular organisms was considered diagnostic, but for smears of foetal stomach contents fluorescent intracellular or extracellular organisms with the morphological characteristics of brucellas were considered significant. Control smears of smooth *Br. abortus* suspensions were included in each series of tests. Blocking tests with unconjugated antiserum to *Br. abortus* were also performed to check the specificity of staining (Cherry, Goldman, Carski & Moody, 1960).

Determination of the specificity of the conjugate

The specificity of the conjugate was checked by staining heat fixed smears of pure cultures of *Aspergillus fumigatus, Bordetella pertussis, Br. abortus* 544, *Br. abortus biotype* 5, *Br. abortus* 45/20, *Br. abortus strain* 19, *Br. canis RM* 6–66, *Br. melitensis* 16 *M, Br. neotomae* 5*K*33, *Br. ovis* 63/92, *Br. suis* 1330, *Campylobacter fetus, Escherichia coli, Francisella tularensis, Listeria monocytogenes, Pasteurella multocida, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus, Yersinia enterocolitica NCTC* 10461, *Y. enterocolitica IX, Y. pseudotuberculosis.*

RESULTS

Unstained smears of bovine placental material from both infected and uninfected sources produced a pronounced yellowish fluorescence in ultra-violet light (Plate 1, fig. 1). This was sufficiently intense to obscure specific fluorescence of microorganisms in infected tissues stained with FITC-labelled antibody. The use of Evans Blue as a counterstain did however enable this difficulty to be overcome. Uninfected tissue and also infected tissue in the absence of fluorescent antibody staining showed a uniform red fluorescence after counter-staining (Plate 1, fig. 2).

Smears of tissue infected with brucella organisms, after fluorescent antibody staining and counterstaining, showed the presence of yellowish-green fluorescent organisms of brucella morphology located in clumps apparently within cells. The surrounding uninfected cells were stained red by the counterstain (Plate 1, fig. 3). These structures were not visible in smears of uninfected tissues stained similarly nor in smears of infected tissue stained with FITC-labelled globulin from unimmunized rabbits. Occasionally in all types of preparation, isolated particles showing yellowish fluorescence or indistinct patches showing similar fluorescence, were observed. However, fluorescent clumps of intracellular organisms were not seen in

Table 1. *Comparison of the results of the direct FAT for* Brucella abortus *with those of conventional tests*

		Results of conventional methods		
Number of samples	Köster's stain	Culture	Animal inoculation	Number positive to FAT = (%)
47	+	*Brucella abortus*	*Brucella abortus*	47 (100)
23	+	*Br. abortus*	ND	23 (100)
11	+	—	ND	7 (63)
7	+	—	*Br. abortus*	6 (85·8)
17	+	—	*Cox. burnetii**	0 (0)
2	+	*Br. abortus*	*Br. abortus + Cox. burnetii**	2 (100)
3	—	*Campylobacter fetus*	— or ND	0 (0)
2	—	*Leptospira* spp.	*Leptospira* spp.	0 (0)
4	—	*Listeria monocytogenes*	*Listeria monocytogenes*	0 (0)
18	—	*Asp. fumigatus*	— or ND	0 (0)
12	—	*Salmonella* spp.	*Salmonella* spp.	0 (0)
5	+	*Salmonella dublin + Br. abortus*	ND	5 (100)
111	—	—	— or ND	2 (1·9)

ND = not done. — = negative or inconclusive.

* Q-fever samples were accumulated over several years and include replicate smears from single animals.

smears of uninfected tissue and such fluorescent clumps were taken as the index of infection in assessing smears stained by FITC-labelled antibody.

Examination of the FITC-labelled anti-*Br. abortus* conjugate showed that it produced bright fluorescence with all of the smooth brucella strains tested, including *Br. abortus, Br. melitensis, Br. neotomae* and *Br. suis*. No staining was observed with *Br. ovis* and rough strains of *Br. abortus* and only weak staining with *Br. canis*. The other organisms tested were not stained by the conjugate with the exception of *Yersinia enterocolitica IX* and *Staph. aureus. Yersinia enterocolitica IX* has not been reported in bovine abortion material as yet, although other *Y. enterocolitica* strains have been detected occasionally. It was included in the present study because of its serological relationship to *Brucella* spp. (Ahvonen, Jansson & Aho, 1969; Corbel & Cullen, 1970). If it should occur it is unlikely to be confused with *Brucella* spp. because of its preferred extracellular location. The reaction of the conjugate with *Staph. aureus* was not simply the result of reaction with antibodies to this organism naturally present in the γ-globulin fraction, as was shown by the fact that the cross-reaction was not eliminated by absorption with *Staph. aureus* without simultaneously removing specific antibody activity and most of the γ-globulin.

A similar reaction was also given by FITC conjugated γ-globulin from uninoculated rabbits. It may have been a result of non-immune reaction between the protein A of *Staph. aureus* and the Fc-component of the γ G-globulins (Forsgren & Sjöquist, 1967). In any event, even though staphylococci are frequently present as contaminants in smears of abortion material, they are unlikely to be confused with

brucellas because of their morphology and location. Furthermore, *Staph. aureus* can easily be differentiated from *Brucella* spp. in smears stained by Gram's method.

Special attention was paid to the reaction of the anti-*Br. abortus*-FITC conjugate with smears of material infected with *Cox. burnetii*. No cross-reaction was obtained with this material and fluorescent intra-cellular organisms were not observed. This suggested that this method could be useful in differentiating brucellosis from Q-fever infection in cases of bovine abortion.

A series of smears of material from abortions for which a diagnosis of *Cox. burnetii* infection had been established by animal inoculation, were examined by the FAT (Plate 1, fig. 4). None of these smears gave a positive reaction to the FAT although all reacted with Köster's stain to give appearances indistinguishable from *Br. abortus* infection. Similarly smears of material from abortions, the cause of which had been determined by culture or microscopy to be infection with *C. fetus, L. monocytogenes, Salmonella* spp., *S. dublin, Leptospira* spp., or *Asp. fumigatus*, all gave negative reactions to the FAT. A small number of positive reactions to the FAT were obtained in smears from material in which infection with *Br. abortus* and other pathogens was simultaneously present.

The results obtained in a series of examinations of material from abortions due to various causes are summarized in Table 1.

DISCUSSION

The principal reason for adopting a fluorescent antibody method for detection of brucellas in smears of bovine abortion material was the necessity for differentiation of *Brucella* spp. from *Cox. burnetii*. Differentiation cannot reliably be obtained by Köster's method and indeed reliance on this may be the source of an incorrect diagnosis when culture or animal inoculation are unsuccessful or impractical. The need for an effective differential test for brucellosis and Q-fever infections under conditions where both are prevalent has been emphasized by Schweizer (1964) and by Lapraik, MacKinnon & Slavin (1967).

The direct FAT for the detection of brucellas in cultures or infected tissues has been studied by Moulton & Meyer (1958); Moody, Biegeleisen & Taylor (1961); Chistov, Pesina & Voronova (1961); Ignat'eva (1961, 1962); Janney & Berman (1962); Biegeleisen, Moody, Marcus & Flynt, (1962); Meyer (1966) and others. However, the application of this test to smears of bovine abortion material is impeded by the pronounced auto-fluorescence often encountered in these samples. The present results showed that this difficulty can be overcome by counter-staining the smears with Evans Blue. Failure to adopt some method of suppressing auto-fluorescence makes interpretation of fluorescent antibody stained smears extremely difficult. Examination of the results obtained in the present study showed that the FAT was at least as sensitive as Köster's method for detecting *Br. abortus* in smears of infected material. A comparison of the results of the direct FAT with conventional staining, culture and animal inoculation gave consistent results. The only smears giving negative reactions to the FAT and positive reactions to Köster's stain were from animals known to have been infected with *Cox. burnetii*.

The number of false positive reactions given by the FAT could not be determined accurately when the results could only be checked by Köster's method. Rather more positive reactions were obtained with the FAT than with Köster's stain but when the results of cultural examination and animal inoculation were available, these confirmed the FAT results.

On smears of placental material, a positive diagnosis was confined to those specimens in which fluorescent clumps of intracellular organisms were visible. On smears of foetal stomach contents, in many instances, more fluorescent extracellular than intracellular organisms were visible and the presence of these was considered diagnostic of *Br. abortus* infection (Plate 1, fig. 5).

Apart from differentiating *Br. abortus* from *Cox. burnetii*, the FAT also successfully differentiated it from *Listeria monocytogenes*, *Salmonella* spp., *C. fetus*, *Leptospira* spp. and fungi. These pathogens are frequently associated with bovine abortion and differentiation is essential, although not likely to be a problem in practice because of the distinctive morphology of these organisms.

These results indicated that the FAT could be of value in the examination of bovine abortion material, particularly when differentiation of brucellas from *Cox. burnetti* was necessary. In such circumstances the FAT should be used in conjunction with the modified Köster's or Ziehl Neelsen procedure. Samples negative to the FAT but positive to the tinctorial stain could then be considered as probably infected with *Cox. burnetii*.

The importance of rapid and accurate identification of *Br. abortus* infection in abortion material is likely to increase as the Brucellosis Eradication Scheme of the Ministry of Agriculture, Fisheries and Food progresses and the incidence of brucellosis declines. Then it will be essential to identify sources of infection as rapidly as possible in order to prevent spread to disease-free animals. In such circumstances the FAT could be of particular value.

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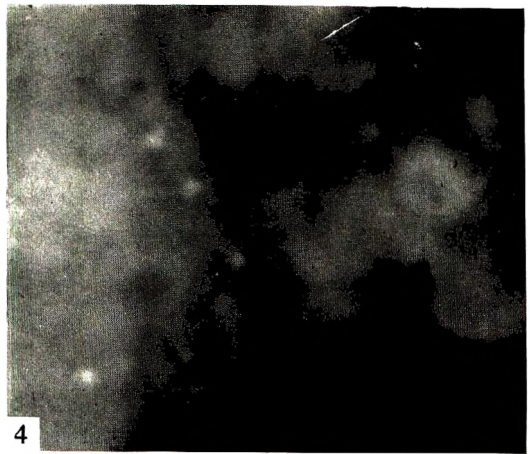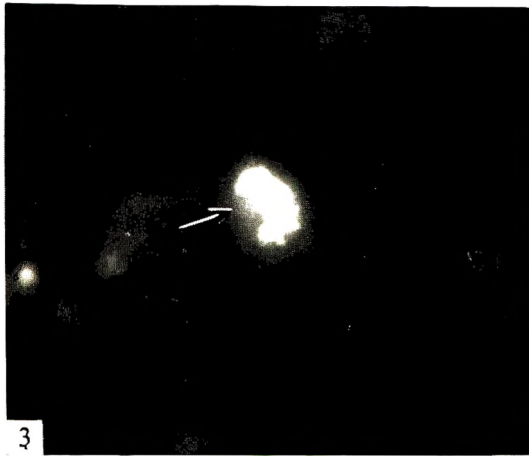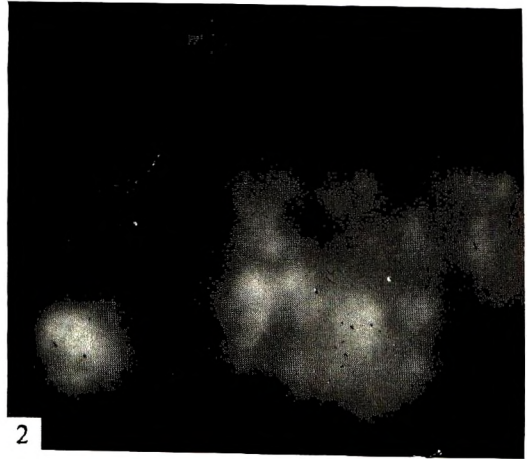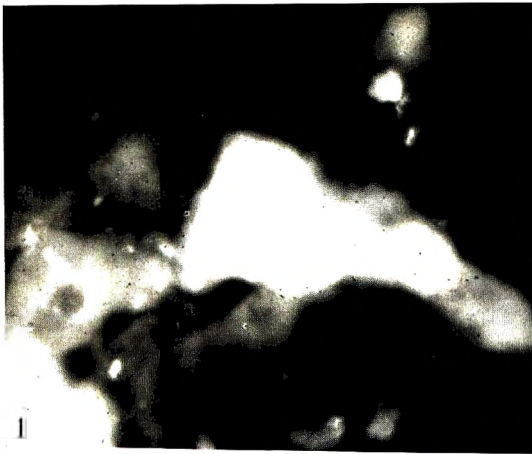
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EXPLANATION OF PLATE

Fig. 1. Unstained smear of bovine placental cotyledon showing intense autofluorescence in ultra-violet light. × 1250.

Fig. 2. Bovine placental smear stained with Evans Blue to suppress autofluorescence. The cytoplasm of the cells was stained red and the nuclei orange-red. × 1250.

Fig. 3. Bovine placental smear infected with *Br. abortus*. This was stained with FITC-labelled antiserum to *Br. abortus* and counterstained with Evans Blue. The brucellas were visible at centre as an intracellular cluster of yellow-green fluorescent organisms (arrowed). × 1250.

Fig. 4. Bovine placental smear infected with *Cox. burnetii* and stained as in Fig. 3. No fluorescent organisms were visible. × 1250.

Fig. 5. Smear of bovine foetal stomach contents infected with *Br. abortus*. The organisms were visible as fluorescent yellow-green clumps of intracellular organisms (arrowed) and as extracellular individual organisms. × 1250.

Studies on two methods for extraction of streptococcal T antigens

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SUMMARY

The extraction of group A streptococcal antigens by group C phage-associated lysin has been confirmed. In addition to the T antigen the extract contained M-protein, group-specific polysaccharide and mucopeptide antigen which was difficult to remove. This method of extraction of the T antigen was compared with the trypsin method. The latter method was found to be of advantage in giving a pure specific antigen.

INTRODUCTION

The serological classification of group A streptococci introduced by Griffith (1934) was established by means of slide-agglutination of intact cells. This reaction depends on the presence of T antigen and hence its use in T-typing of these streptococci.

However, precipitation reactions of T antigens were only practised on a small scale and the only available method of extraction of these antigens was by treatment with trypsin after heating the cells to a temperature of 70° C. (Lancefield & Dole, 1946; Pakula, 1951; McLean, 1953). Recently, Kantor & Cole (1960) reported the extraction of type 1 T antigen by means of group C streptococcal phage-associated lysin (Maxted, 1957; Krause, 1957).

In this communication results of studies aiming at confirming previous findings with the phage-associated lysin extraction method and comparing it with the already established method of extraction with trypsin are reported. T antigens of three T-types other than type 1 (types 2, 5 and 12) were extracted with this lysin.

MATERIALS AND METHODS

Strains of streptococci

The following four T types of group A streptococci were used in the investigation:

(1)	N.C.T.C.	100079	type 1
(2)	N.C.T.C.	8322	type 2
(3)	N.C.T.C.	100080	type 5
(4)	N.C.T.C.	100081	type 12

All cultures were grown in 2 litre amounts of Todd–Hewitt broth (Oxoid) for 3 days in the air at 30° C.

Preparation of the streptococcal cell walls

The cultures were killed by heating in a water-bath at 56° C. for $\frac{1}{2}$ hr. The cells were then washed three times in sterile normal saline. Cell walls were prepared from the washed killed cells in a Mickle's disintegrator as described by Salton & Horne (1951).

Antisera

Group A streptococcal typing antisera (T and M specific antisera) and grouping antisera (group A specific and group A-variant specific antisera) were prepared as previously described (Erwa, Maxted & Brighton, 1969). The antisera used for typing by the agglutination method, T-typing, contain only the T-type antibody, while those used for M-typing contain both M and T antibodies.

Preparation of antigens

T antigen

This was prepared by two different methods as follows:

Trypsin method (Lancefield & Dole, 1946; Pakula, 1951). The streptococcal cells, killed at 70° C., were suspended in a suitable volume of phosphate buffer pH 7·8, and treated with 0·5 % crystalline trypsin at 37° C. for a few hours. The trypsinized suspension was then centrifuged and the supernatant fluid collected. This was acidified by N-HCl to pH 2·5 at which maximum precipitation of the T protein occurred. The precipitate was allowed to settle in the refrigerator overnight and then collected by centrifugation. It was then redissolved in a minimum amount of phosphate-buffered saline pH 7·2 and used in serological tests.

Extraction by group C phage-associated lysin. This muralytic enzyme was obtained by propagating the bacteriophage on group C streptococci (Maxted, 1957; Krause, 1957) grown in Todd–Hewitt broth. The lysin was reduced by the addition of a few drops of thioglycollic acid neutralized to pH 7·4 with N-NaOH. Twenty ml. amounts of the lysin were used to lyse cell walls from 2 litre cultures. The suspensions of cell walls in lysin were incubated at 37° C. for a few hours. The cell walls were then separated from the suspension by centrifugation at 10,000 rev./min. for 30 min. The supernatant fluid was taken as the crude extract. One half of the extract was treated with trypsin in order to destroy M-protein present. The other half was left untrypsinized.

For purification, each of the trypsinized and the untrypsinized parts of the crude extract was then divided into two lots. One lot was purified by precipitation of the protein at pH 2·5 by adding N-HCl dropwise. The precipitate was then redissolved in water and reprecipitated by acid and the addition of 2·5 volumes of absolute alcohol. The latter would remove any group-specific polysaccharide in the extract (Fuller, 1938). This procedure was repeated four or five times.

The second lot was purified by precipitation with saturated ammonium sulphate. Three different concentrations of ammonium sulphate were used, 50 %, 60 % and 70 %, and the extracts were contained in dialysis bags suspended in these solutions. This process continued overnight at refrigerator temperature (1–4° C.). The deposits were then collected by centrifugation, redissolved in water and dialysed in dis-

tilled water in the cold with several changes of water. The solution was then acidified to pH 2·5 and washed in absolute alcohol as before. The purified deposits were redissolved in a minimum amount of phosphate-buffered saline pH 7·2 and used for serological tests.

Group-specific polysaccharide

Group A and group A-variant specific polysaccharides were prepared from cell walls by the hot formamide method of Fuller (1938) and purified by several treatments with acid-alcohol and acetone as previously described (Erwa *et al.* 1969).

Mucopeptide

The formamide residue obtained after the extraction of the group-specific polysaccharide was treated with crystalline egg lysozyme solution (0·5 mg/ml) in citrate buffer pH 5·3 (Krause & McCarty, 1961). The pH was adjusted to 7·2 by N-NaOH and used for the serological tests.

Serological tests

The presence of T antigens was tested for by the capillary precipitation test of Swift, Wilson & Lancefield (1943). Group-specific precipitation reactions and mucopeptide reactions were detected by the ring precipitin technique of Lancefield (1933).

Absorption of group-specific antibodies and mucopeptide antibodies was carried out by adding equal volumes of extract and antiserum, allowing the absorption to continue at 37° C. for 1 hr. and then overnight at refrigerator temperature. The precipitate was separated by centrifugation and discarded.

RESULTS

Extraction of the T antigen by the trypsin method

Group A streptococci of T types 1, 2, 5 and 12 were extracted by trypsin treatment and purified as described above. They were then tested by the capillary precipitation method and ring precipitin technique. In the former method homologous and heterologous T and M antisera were used. In the latter, group A antiserum (grouping serum) was used in order to exclude any group reaction. The results are shown in Table 1. The extracts reacted specifically with their homologous T and M antisera and showed no cross-reactions with the control heterologous antisera. The reaction of the M antisera with T extracts of homologous types is explained by the fact that M antisera contain T antibodies as well since absorption is carried out to remove group-specific antibodies only. On the other hand there were no reactions with the grouping serum (group A antiserum) when tested with the extracts.

Extraction of the T antigen by the phage-associated lysin

Crude group C streptococcal phage-associated lysin was used to extract T antigens from cell walls of group A streptococci types 1, 2, 5 and 12 as described previously. The crude extract obtained from each type was divided into two parts.

Table 1. *Precipitation reactions of purified trypsin-extracted T antigens of types* 1, 2, 5, 12 *group A streptococci with homologous and heterologous T and M specific antisera and with group A specific antiserum*

T extract	Type 1		Type 2		Type 5		Type 12		Reactions with group A antiserum
	T 1	M 1	T 2	M 2	T 5	M 5	T 12	M 12	
Type 1	3+	4+	−	−	−	−	−	−	−
Type 2	−	−	2+	2+	−	−	−	−	−
Type 5	−	−	−	−	2+	4+	−	−	−
Type 12	−	−	−	−	−	−	2+	3+	−

− = no reaction.
± = trace, precipitation column < 0·25 cm.
1+ = weak, precipitation column 0·25–0·5 cm.
2+ = moderate, precipitation column 0·5–1·0 cm.
3+ = strong, precipitation column 1·0–1·5 cm.
4+ = very strong, precipitation column > 1·5 cm.

Table 2. *Results of precipitation reactions of phage lysin extracts of group A T types* 1, 2, 5 *and* 12 *streptococci with T and M type-specific antisera as well as group A specific antiserum*

T antigen		T antisera				M antisera				Reactions with grouping antiserum
		T 1	T 2	T 5	T 12	M 1	M 2	M 5	M 12	
Type 1 extract	i	+	−	−	−	+	−	−	−	4+
	ii	±	−	−	−	+	−	−	−	4+
	iii	2+	−	−	−	2+	−	−	−	4+
	iv	2+	−	−	−	2+	−	−	−	4+
Type 2 extract	i	−	2+	−	−	−	±	−	−	4+
	ii	−	2+	−	−	−	±	−	−	4+
	iii	−	±	−	−	−	+	−	−	3+
	iv	−	+	−	−	−	2+	−	−	2+
Type 5 extract	i	−	−	±	−	−	−	±	−	4+
	ii	−	−	+	−	−	−	±	−	3+
	iii	−	−	+	−	−	−	+	−	4+
	iv	−	−	±	−	−	−	+	−	4+
Type 12 extract	i	−	−	−	+	−	−	−	+	4+
	ii	−	−	−	+	−	−	−	±	4+
	iii	−	−	−	+	−	−	−	±	4+
	iv	−	−	−	2+	−	−	−	+	4+

(i) Trypsinized crude extract.
(ii) Trypsinized acid-precipitated extract.
(iii) Untrypsinized crude extract.
(iv) Untrypsinized acid-precipitated extract.
For grades of the type-specific reactions see footnote for Table 1, and for group-specific reactions see footnote for Table 4.

Table 3. *Precipitation reactions with T antigen extracted by means of phage lysin and subsequently digested with trypsin and precipitated with saturated ammonium sulphate*

T Antigen		T 1	T 2	T 5	T 12	Reactions with group A antisera
Type 1	X	+	−	−	−	3 +
	Y	+	−	−	−	3 +
	Z	+	−	−	−	2 +
Type 2	X	−	+	−	−	3 +
	Y	−	+	−	−	3 +
	Z	−	+	−	−	2 +
Type 5	X	−	−	+	−	3 +
	Y	−	−	+	−	2 +
	Z	−	−	+	−	2 +
Type 12	X	−	−	−	+	3 +
	Y	−	−	−	+	3 +
	Z	−	−	−	+	3 +

(Reactions with T antisera span T 1, T 2, T 5, T 12.)

X = trypsinized extract precipitated with 50 % $(NH_4)_2SO_4$.
Y = trypsinized extract precipitated with 60 % $(NH_4)_2SO_4$.
Z = trypsinized extract precipitated with 70 % $(NH_4)_2SO_4$.

One part was treated with trypsin to destroy any M-protein present. The other part was left without treatment. A volume of each of the two parts was then acidified to pH 2·5 in order to precipitate the T antigen. Thus four lots of the extract resulted: (i) trypsinized crude extract, (ii) trypsinized acid-precipitated extract, (iii) untrypsinized crude extract and (iv) untrypsinized acid-precipitated extract. These four lots were tested serologically by the capillary precipitation test with homologous T and M antisera. They were also tested for group reaction with group A specific antiserum. The results are shown in table 2. All the four preparations reacted with the homologous T antisera without cross-reactions with the heterologous antisera. Reactions with M antisera of homologous types persisted even after removal of M-proteins by trypsin and this is due to the presence of T antibodies in the M antisera as explained above. Therefore, the four lots of type 1 extract reacted with T 1 and M 1 antisera, the four lots of type 2 with T 2 and M 2 antisera, etc.

However, all extracts reacted strongly with grouping sera, i.e. even after precipitation with acid and washing with alcohol (Table 2). In view of this, it was considered necessary to purify the extracts in such a way that the protein antigen would be completely separated from the carbohydrate antigen. The crude extracts were therefore first trypsinized and then treated with saturated ammonium sulphate solution to final concentrations of 50, 60 and 70 %. The T antigen preparations so obtained were labelled X, Y and Z respectively and tested as before. The results are set out in Table 3. As before there were no cross-reactions with T heterologous antisera but again the group reaction persisted and only slightly diminished in intensity (from 4+ to 2+ or 3+).

However, the reactions of the extracts with the grouping serum could be, to

Table 4. *Results of precipitation reactions of T antigens obtained from trypsinized phage-lysin extracts by treatment with saturated ammonium sulphate and acid* (pH 2·5) *and tested against streptococcal group A and group A-var. antisera before and after absorption with their respective group-specific polysaccharides*

		Precipitation reactions of T antigen extracts							
		Type 1		Type 2		Type 5		Type 12	
Antiserum		a	b	a	b	a	b	a	b
Group A antiserum	Before absorption	3+	4+	3+	3+	4+	3+	3+	4+
	After absorption	3+	2+	2+	+	3+	2+	3+	3+
Group A-var. anti-serum	Before absorption	3+	3+	3+	3+	3+	3+	3+	3+
	After absorption	3+	3+	3+	2+	3+	2+	2+	2+

a = T antigen precipitated by saturated $(NH_4)_2SO_4$.
b = T antigen precipitated by acid (pH 2·5).
− = negative reaction.
± = trace reaction after 2 hr.
+ = weak reaction after 1–2 hr.
2+ = moderate reaction after $\frac{1}{2}$–1 hr.
3+ = strong reaction after 5–30 min.
4+ = very strong reaction after < 5 min.

a large extent, due to the presence of mucopeptide in the extracts. Streptococcal mucopeptides are strongly antigenic (Abdulla & Schwab, 1965; Karakawa, Lackland & Krause, 1966), and antibodies to them are invariably found in sera prepared by hyperimmunization with streptococcal cells or cell walls of which they are a constituent. Therefore, precipitation tests of the extracts were repeated with group A antiserum and with the heterologous group A-var. antiserum as shown in Table 4 (the reactions before absorption with group-specific polysaccharides). Both antisera gave positive results, although the reactions with group A-var. were rather slow to appear, i.e. after 5–30 min. This finding suggests that the reaction is not due to group A polysaccharide. To confirm this, small quantities of group A and group A-var. antisera were absorbed with purified group A and group A-var. polysaccharides (Erwa et al. 1969) and retested as above. The results are also included in Table 4, and it is evident that the reactions with the group antisera persisted even after absorption with the respective group-specific polysaccharide. Generally the intensity of precipitation after absorption either remained unchanged or fell only slightly. Only with types 1 and 2 was there an appreciable fall.

Furthermore, a mucopeptide extract prepared as described in 'Materials and Methods' above was used for the absorption of homologous antibodies from group A and group A-var. antiserum. The absorbed serum showed effective loss of reaction with the T antigen preparations when tested by the precipitation technique as shown in Table 5. The reactions of the T antigens with the mucopeptide-absorbed sera were almost equal to those given by the mucopeptide and the same absorbed sera (1+ or ±).

Table 5. *Precipitation reactions of T antigens obtained from trypsinized phage-lysin extracts by treatment with saturated ammonium sulphate and acid (pH 2·5) and tested against groups A and A-var. antisera before and after absorption with a streptococcal mucopeptide*

Antiserum		Type 1		Type 2		Type 5		Type 12		Reactions with the mucopeptide extract
		a	b	a	b	a	b	a	b	
Group A antiserum	Before absorption	3+	4+	3+	4+	4+	3+	3+	3+	3+
	After absorption	±	1+	±	1+	1+	±	1+	±	±
Group A-var. antiserum	Before absorption	3+	3+	3+	3+	3+	3+	3+	3+	3+
	After absorption	±	±	1+	±	±	±	1+	±	−

N.B. for explanation of signs see footnote for Table 4.

In summary the absorption tests carried out generally showed the failure of effective reduction of the intensity of reactions of the T extracts with the grouping sera when the latter were absorbed with purified polysaccharides. On the other hand, with the mucopeptide extract the reaction was almost abolished.

DISCUSSION

The results presented above demonstrate clearly the effectiveness of group C phage-associated lysin in releasing the T antigen from the streptococcal cell wall. The soluble antigen so obtained is readily detectable by precipitation reactions indicating a satisfactory serological reactivity.

However, extracts with phage-associated lysin present certain difficulties. The main difficulty encountered in our work was the release of other cellular antigens alongside the T antigen. The group-specific polysaccharide is one (Maxted, 1957; Krause, 1957), but its removal was easy as shown above. The M-protein is another such antigen (Kantor & Cole, 1960) and again we were able to get rid of it by trypsin treatment. However, the greatest challenge came with the attempts to remove the mucopeptide antigen which was found to be present in the extract. In this respect efforts to separate the mucopeptide from the T antigen were not fruitful and its serological reactivity persisted in all extracts.

Furthermore, the phage-associated lysin is rather unstable and has to be used in the reduced condition and like other enzymes it requires adjustment of the medium to optimal pH and temperature in order to give a satisfactory reaction.

On the other hand the T antigen extracted by the trypsin method (Lancefield & Dole, 1946; Pakula, 1951) gave highly specific reactions with homologous antisera and showed no cross-reactions of any sort connected with any of the four types of group A streptococci investigated here, thus indicating the lack of other contaminating cellular antigens. The trypsin destroys the M-protein. The group-

specific polysaccharide needs a more vigorous treatment for its release, such as hot formamide (Fuller, 1938), hot acid (Lancefield, 1933), muralytic enzymes (Maxted, 1948, 1957). Nevertheless trace amounts of polysaccharide which might be found in extracts made by trypsin digestion are easily removed after precipitation of the T antigen at pH 2·5 and washing with absolute alcohol. Moreover, since the cells remain intact after trypsin treatment, the mucopeptide antigen is not released in the extract. In general all of these facts endow the trypsin method extract with an advantage, since it is easy to prepare and purify as well as being highly specific in serological reactions. In our opinion this method of T antigen extraction is to be recommended for all practical purposes requiring serological purity and specificity of the antigen.

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On the aetiology of whooping cough

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SUMMARY

The results of routine bacterial and viral studies on 483 patients with whooping cough, investigated at one hospital over a five year period are presented. The possible role of respiratory viruses in the aetiology of the disease is discussed.

INTRODUCTION

A number of studies have suggested that viruses may cause whooping cough.

In 1934 McCordock & Smith described inclusion bearing cells in the epithelium of the upper respiratory tract of children who died of pneumonia following whooping cough. The inclusions which they described resemble those produced by some viruses.

Following the introduction of tissue culture techniques, Olson, Miller & Hanshaw (1964) isolated adenovirus type 12 from the throats of four children with whooping cough. Infection was confirmed by subsequent rises in neutralizing antibodies, and in each patient cultures for *Bordetella pertussis* were negative. In a prospective study conducted by Edinburgh City Hospital, Urquhart, Moffat, Calder & Cruickshank (1965) recovered viruses, a third of which were adenoviruses, from 15 of 49 children with clinical whooping cough. *B. pertussis* was isolated from only two of the 49 patients. In 1966 Collier, Connor & Irving isolated adenovirus type 5 from the liver, lungs and kidney of a 4-year-old child who died following a whooping cough-like illness complicated by pneumonia. Connor (1970) isolated adenoviruses (type 1, 2, 3 and 5) from 11 of 13 sporadic cases of whooping cough in which there was no evidence of infection with *B. pertussis* by culture or serology.

Pereira & Candeias (1971) isolated 37 strains of virus, almost a third of which were adenoviruses, from 136 Brazilian children with whooping cough. Although *B. pertussis* was recovered from 29 children, the virus isolation rate was twice as high in the group from whom *B. pertussis* was not cultured.

About the same time, Sturdy, Court & Gardner (1971) reported on 34 children with whooping cough admitted to hospitals in the Newcastle area. *B. pertussis* was not isolated from any of these children, whereas viruses, mainly respiratory syncytial virus and adenovirus, were obtained from 20. These authors suggested that, in view of the doubt existing about the aetiology of whooping cough, a large-scale prospective study should be undertaken.

The results presented in this paper review 5 years experience of routine bacterial and viral studies performed on patients with whooping cough at Fairfield Hospital for Communicable Diseases, Melbourne.
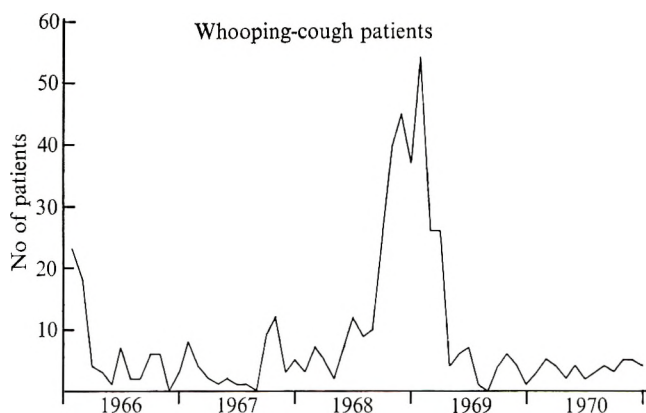
Fig. 1. The number of patients admitted to Fairfield Hospital for Communicable Diseases with whooping cough from 1 January 1966 to 31 December 1970.

PATIENTS AND METHODS

Epidemics of whooping cough occur in Melbourne every three or four years, particularly during the spring and summer months (see Fig. 1) and are reflected by the admission of large numbers of patients with this disease to Fairfield Hospital.

All patients with respiratory illnesses admitted to this Hospital are nursed in single rooms until a diagnosis is made. Two throat swabs, one for bacterial, the other for viral culture, are taken from each patient by the admitting doctor.

During this study throat swabs for viral studies were collected and processed as previously described (Kennett, Ellis, Lewis & Gust, 1972). The resulting material was inoculated into duplicate tubes of primary cynomolgus monkey kidney, HeLa and human embryonic fibroblast cell cultures. Standard procedures were used for the identification of strains isolated. In patients with suspected whooping cough an additional specimen was obtained for bacterial study by swabbing the nasopharynx with a cotton-wool swab introduced through the nose on a long flexible wire. These swabs were transported to the laboratory promptly and immediately plated out on horse blood agar and a second medium chosen to facilitate the growth of *B. pertussis*. Before June, 1968, meat extract medium (Nicholson & Turner, 1954) with 20 % citrated sheep's blood was used, but subsequently this has been replaced by Bordet-Gengou (Oxoid) containing 20 % defibrinated sheep's blood,* penicillin 0·35 units/ml. and no glycerol. All media were incubated aerobically at 37° C. and held for 5–7 days.

RESULTS

The results obtained over the 5-year period from 1 January 1966 to 31 December 1970, are presented. This period included a large whooping cough epidemic (see Fig. 1).

During the five years 502 patients with clinical whooping cough were admitted to Fairfield Hospital for Communicable Diseases, Melbourne. Throat swabs for

* Commonwealth Serum Laboratories, Melbourne.

Table 1. *The number of patients with whooping cough, admitted to Fairfield Hospital over a 5-year period, from whom* Bordetella pertussis *and virus strains were isolated*

Year	No. of patients	No. from whom B. pertussis isolated	No. from whom a virus or viruses isolated
1966	70	13 (18·6 %)	9 (12·8 %)
1967	36	11 (30·6 %)	12 (33·3 %)
1968	199	60 (30·2 %)	46 (23·1 %)
1969	134	70 (52·2 %)	34 (25·4 %)
1970	44	21 (47·7 %)	15 (34·0 %)
Total	483	175 (36·2 %)	116 (24·0 %)

Table 2. *The isolation rate of* Bordetella pertussis *from patients with whooping cough, related to the patient's age*

Age	No. of patients B. pertussis		
	Isolated	Not isolated	Isolation rate (%)
< 1 year	94	144	39·5
1–2 years	36	30	54·5
> 2–3 years	17	32	34·7
> 3–4 years	13	24	35·1
> 4–5 years	6	24	20·0
> 5–10 years	7	47	13·0
> 10 years	2	7	22·2
Total	175	308	36·2

viral culture and nasopharyngeal swabs for bacterial culture were obtained from 483 patients. The nineteen patients from whom both types of swab were not received have been excluded from the study.

The isolation rate of *B. pertussis* was low in 1966–68 but in the last 2 years has been approximately 50 % (see Table 1).

The isolation rate was higher in babies and younger children (see Table 2) who are usually admitted to hospital earlier in their illness (N. McK. Bennett, in preparation).

A total of 132 strains, representing 22 different viruses were recovered from 116 of the 483 patients (see Tables 3 and 4).

The virus isolation rate was similar whether *B. pertussis* was also isolated or not (see Table 5).

Adenoviruses (types 1, 2, 5, 6, 7 and 9) were the most commonly encountered group, and were isolated from 49 (42 %) of the 116 patients from whom virus was recovered.

As viral studies are routinely performed on all patients with respiratory disease admitted to this hospital, the relative isolation rate provides an index of their occurrence in the community. During the 11-year period, 1 January 1960 to 31 December 1970, 513 strains of adenovirus (types 1–7, 9, 10 and 15) were isolated from 7267 patients with respiratory disease (including whooping cough). The monthly isolations are compared with the number of admissions of patients with whooping cough during the same period (see Fig. 2).

Table 3. *The strains of virus recovered from patients with whooping cough*

Virus	B. pertussis		Total
	Isolated	Not isolated	
Single isolation			
Adeno. (1, 2, 5, 6, 7)	18	20	38
Influenza (A and B)	1	1	2
Para-influenza (1, 2, 3)	3	7	10
Rhino.	5	8	13
Respiratory syncytial	1	8	9
Coxsackie (A 9, B 1, B 4)	1	4	5
Herpes hominus	4	5	9
Cytomegalo.	3	7	10
Miscellaneous enteroviruses (ECHO 6, 22, Polio. 2, ENT.*)	3	2	5
Multiple isolations (see Table 4)	10	21	31
Totals	49	83	132

* Enterovirus not typed.

Table 4. *Viruses involved in multiple isolations*

B. pertussis isolated	B. pertussis not isolated	
Adeno. 1:H. hominus	Adeno. 2:Paraflu. 1	Adeno. 9:ENT.*
Adeno. 1:Paraflu. 3	Adeno. 2:Rhino.	Influenza A 2:R.S.V.†
Adeno. 2:Rhino.	Adeno. 2:H. hominus	Influenza B:Echo 11
Adeno. 5:C.M.V.	Adeno. 2:Polio. 2	H. hominus:polio. 2
Adeno. 5:Polio. 2	Adeno. 7:Paraflu. 2	Paraflu. 3:R.S.V.:H. hominus

* ENT. = enterovirus, not typed.
† R.S.V. = Respiratory syncytial virus.

Table 5. *The isolation rate of viruses from patients with whooping cough from whom* Bordetella pertussis *was and was not isolated*

	No. of patients	No. from whom a virus or viruses isolated	No. from whom an adenovirus isolated
B. pertussis isolated	175	44 (25·1 %)	23 (13·1 %)
B. pertussis not isolated	308	72 (23·4 %)	26 (8·4 %)
Total	483	116 (24·0 %)	49 (10·1 %)

DISCUSSION

In recent years there has been a tendency to emphasize the importance of viruses in the aetiology of whooping cough and to diminish the role of *B. pertussis*. The reason for this altered emphasis is not hard to find. The isolation rate of *B. pertussis* from patients with whooping cough has often been disappointingly low and this had led to a search for other possible agents, in particular viruses.

In this study the results of routine bacterial and viral studies on 483 patients with whooping cough investigated at Fairfield Hospital over a 5-year period are analysed.
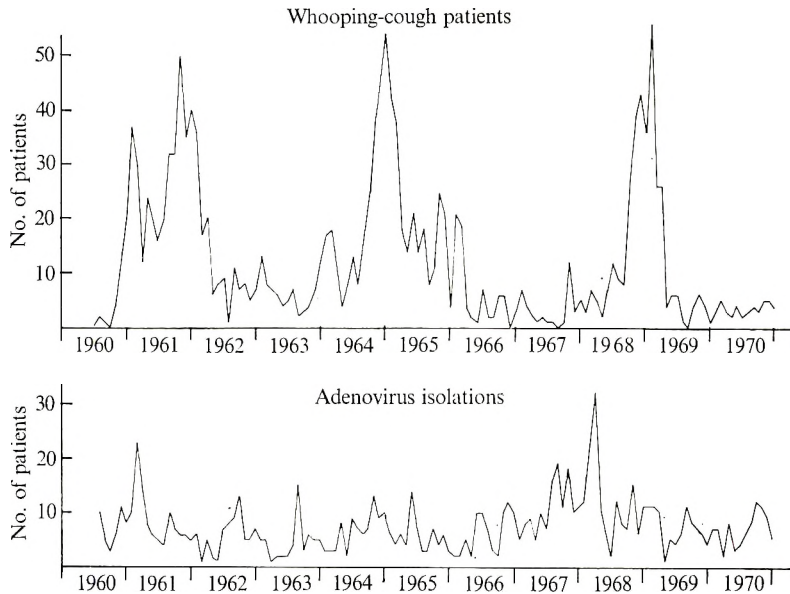
Fig. 2. The monthly admissions of patients with whooping cough to Fairfield Hospital for Communicable Diseases from January 1960 to December 1970, compared with the monthly isolations of adenoviruses from all patients with respiratory disease seen at the same hospital over this period.

The isolation rate of *B. pertussis*, 36·2 %, compares favourably with other series (A combined Scottish study, 1970; Pereira & Candeias, 1971), and after the introduction of Bordet–Gengou medium in mid-1968 it increased to approximately 50 %. This figure probably underestimates the proportion of infections due to *B. pertussis* as usually only one swab was taken from each patient and no serological investigations were performed.

As *B. pertussis* is only capable of surviving for a few hours *in vitro* in nasal secretions, isolation attempts require considerable co-operation between clinicians and laboratory staff. The different isolation rates found in this series probably represent variations in efficiency and frequency of swabbing, delays in plating out and differences in the type and age of culture media used.

In this study one or more respiratory viruses were recovered from the throat of 24 % of patients. The techniques used were effective for the isolation of all the common respiratory viruses, except coronaviruses, and over the 5-year period a wide range of strains was obtained. In common with other series the group most commonly encountered were adenoviruses.

If, as is postulated, respiratory viruses have an important role in the aetiology of whooping cough, it would be expected that they would be isolated more frequently from patients from whom *B. pertussis* was not grown. In our series there was no significant difference between the viral isolation rate in the two groups ($P > 0.05$).

Table 6. *A statistical analysis of the relationship between the isolation of virus and of* Bordetella pertussis *from patients with whooping cough investigated over a 5-year period*

	Virus isolated	Virus not isolated	Total
B. pertussis isolated:			
Observed	44	131	175
Expected	42	133	
B. pertussis not isolated:			
Observed	72	236	308
Expected	74	234	
Total	116	367	483

$$\chi^2 = 0.11; \text{d.f.} = 1; P > 0.05.$$

Adenoviruses are often proposed as aetiological agents in whooping cough. In this series there was little difference in the isolation rate of adenoviruses from patients in whom *B. pertussis* was, or was not, isolated. In addition inspection of Fig. 2 fails to reveal a correlation between the number of patients admitted to hospital with whooping cough and the prevalence of adenovirus infections in the community.

Although viruses may be implicated in occasional cases of whooping cough, there is no evidence to suggest that they are responsible for a large proportion of cases.

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Social class and infectious mononucleosis

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SUMMARY

The socio-economic status of 80 patients with infectious mononucleosis was compared with the socio-economic distribution of the general population in the same area of south-west London. An excess incidence of infectious mononucleosis was observed among subjects from upper socio-economic groups. A possible relationship between this observation and the epidemiology of the Epstein–Barr virus is discussed.

INTRODUCTION

Students in the U.S.A. have a high incidence of infectious mononucleosis (Evans, 1960; Evans, Niederman & McCollum, 1968), and similarly students in the United Kingdom appear to be particularly susceptible, although few prevalence studies have been reported from this country. Little is known of the incidence of the disease in other occupational groups, partly because of the low incidence of infectious mononucleosis (IM) in the general population. According to laboratory reports from England and Wales, the incidence of IM is highest among doctors, nurses and other medical workers, and lowest among manual workers (Newell, 1957). Similar findings have been reported in the U.S.A. Niederman (1956) compared the occupations of patients admitted to hospital for IM with the occupations of those admitted for some other diseases. Thirty-two per cent of IM patients were hospital workers compared with only 4% of patients with other diseases. In non-medical personnel IM was equally common in manual and professional workers.

Unfortunately the results obtained in both these surveys are likely to have been influenced by the relative ease with which persons in different occupations have access to diagnostic facilities. The aim of the present study was, therefore, to monitor all cases of IM within a defined mixed residential area, and to relate the socio-economic status of these patients to the social structure of the population from which they were drawn.

PATIENTS AND METHODS

The population studied was that of the immediate catchment area of St George's Hospital, London, S.W. 17, which covers fifteen municipal wards of two Greater London boroughs; the total population of the area at the 1966 10% census was 225,620. The investigation formed part of a wider survey of the sero-epidemiology of the Epstein–Barr virus.

Patients with IM were detected in three ways.

(i) Notifications by general practitioners in the area.

(ii) Requests to the hospital haematology laboratory for Paul–Bunnell tests.

(iii) Admissions to hospital.

Patients with a history and physical signs compatible with IM were admitted to the study if they developed a positive Paul–Bunnell–Davidsohn differential absorption test. Most patients also had an atypical lymphocytosis in the peripheral blood. Students and nurses living in institutions were excluded from the study.

The social class analysis was based on 80 of 92 consecutive cases of IM detected in the catchment area during the period from 1 November 1969 to 13 February 1971. Because many of the patients were still at school, the social background of the IM group as a whole was assessed by defining in each case the socio-economic status of the male head of the patient's household. Seventy-one families (containing 76 index cases) were visited, and each was allotted on this basis to one of 17 socio-economic groups (General Register Office, 1966). The socio-economic background of a further four patients (all adult males living away from home) was assessed on the basis of the patient's own occupation.

Seven of the patients omitted from the analysis could not be accurately classified using the information available; the remaining five patients were excluded because the lack of a male head of household prevented subsequent comparison with the catchment area population. Occupational information was sufficiently complete to indicate that four of the twelve patients omitted were from upper, and two from lower, socio-economic groups.

The social structure of the catchment area population was obtained from the 1966 10% census, which gave information for each of the 15 municipal wards involved. These data provided the socio-economic distribution of economically active males; the distribution of male heads of household was assumed to be the same.

RESULTS

Comparisons between the IM population and the catchment area population (see Table 1) revealed an excess of IM subjects in each of three occupational categories made up of non-manual workers, and a deficit in each of four occupational categories mostly containing manual workers. The excess of IM patients in upper socio-economic groups ('Group A') and the corresponding deficit in lower socio-economic groups ('Group B') was statistically significant ($\chi^2 = 8.6$, $P < 0.01$).

DISCUSSION

The observed differences in the incidence of infectious mononucleosis may have been influenced by under-diagnosis among members of lower socio-economic groups, since such subjects may have been less frequently investigated and may have been less likely to consult a doctor in the first place. On the other hand, the general practitioners in the area were asked to notify all cases of *suspected* infectious mononucleosis (and such patients were often found to have other illnesses) so it is unlikely that many cases reaching medical attention were missed. This is sub-

Table 1. *Comparison of the socio-economic distribution of IM patients with that of the surrounding general population*

Socio-economic groups	Occupational category	Economically active males in population (1966 10% census)		Infectious mononucleosis patients (by male head of household)*	
		Nos.	%	Nos.	%
'Group A'					
1, 2, 13	Employers and managers	679	9·7	12	16
3, 4	Professional workers	306	4·4	4	4
5, 6	Non-manual workers (skilled and partly skilled)	1761	25·3	26	34
Total group A	—	2746	39·4	42	55
'Group B'					
8, 9	Skilled manual workers	2335	33·5	23	31
7, 10, 15	Semi-skilled manual workers / Personal service workers	1028	14·7	5	7
12, 14	Own-account workers (other than professional)	311	4·5	3	4
11, 16, 17	Unskilled manual workers	553	7·9	2	3
Total Group B	—	4228	60·6	33	45
Total all subjects		6974	100	75	100

* See text.

stantiated by the total incidence in the catchment area ($3 \cdot 3$ cases per 10,000 population per annum) which was similar to that quoted for other areas of the United Kingdom (reviewed by Pollock, 1969).

The results may also have been influenced by undetected differences between the two populations. It was assumed, for example, that the socio-economic distribution of economically active males in the catchment area would accurately reflect that of male heads of household, but it is possible that single men without dependents formed a relatively larger proportion of this population in some categories, e.g. unskilled manual workers. This would tend to 'weight' the catchment area population in favour of lower socio-economic groups, though it seems unlikely that the results can be entirely accounted for on this basis.

The prevalence of IM among the families of professional workers did not reflect the overall excess observed in upper socio-economic groups. However, the incidence of IM among young adults from upper socio-economic backgrounds was probably underestimated, because a high proportion of such subjects tend to be students living away from home. If it had been possible to detect IM among the absent student members of families living in the catchment area, the excess of IM in upper socio-economic groups would probably have been greater. No attempt was made to detect IM among students and nurses living in institutions, since such subjects did not form part of the 'general' population of the catchment area.

The observed socio-economic distribution of infectious mononucleosis should be contrasted with that of antibody to the Epstein–Barr virus, an agent considered by many to be the 'necessary cause' of the disease. Thus children in the U.S.A. from middle-class backgrounds have a low prevalence of antibody (Henle & Henle, 1970) while a high antibody prevalence has been reported in lower socio-economic groups, e.g. military recruits (Lehane, 1970), paediatric patients in Philadelphia (Henle & Henle, 1967) and the mixed population of Chicago (Tischendorf *et al.* 1970). The observation that IM is more common among upper socio-economic groups therefore supports the idea that the disease results from a primary Epstein–Barr virus infection in subjects who have escaped subclinical infection in childhood.

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Epstein–Barr virus antibody in cases and contacts of infectious mononucleosis; a family study

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SUMMARY

Serological investigations were carried out on 147 patients with Paul–Bunnell positive infectious mononucleosis (IM) from the general population. All possessed antibody to the Epstein–Barr virus (EBV) and 63 % showed serological evidence of recent infection. Contacts of 132 patients, 306 in all, were followed serologically; within 6 months of the index cases' illness twenty one contacts developed evidence of EBV infection or re-infection and of these five developed overt IM. The secondary attack rate of EBV infection among susceptible contacts was at least 19 %; the corresponding figure for clinically apparent IM was 6 %. EBV antibody prevalence among patients' siblings was significantly lower than among age-matched controls, suggesting that cases of IM come from families with a lower than normal previous experience of the virus. Of thirteen patients with persistently Paul–Bunnell negative 'glandular fever' four had serological evidence of recent EBV infection and two had probable cytomegalovirus mononucleosis. Recent EBV infection may have been associated with the illnesses of five of the remaining patients.

INTRODUCTION

Although the aetiology of infectious mononucleosis (IM) is not firmly established, there is now a considerable amount of evidence incriminating the Epstein–Barr virus, a herpes-like agent, as the causative organism. The Epstein–Barr virus (EBV) has been particularly associated with Burkitt's lymphoma (Henle *et al.* 1969; Epstein, 1970), nasopharyngeal carcinoma (Henle, Henle & Diehl, 1968), sarcoidosis (Hirshaut *et al.* 1970), systemic lupus erythematosus (Evans, Rothfield & Niederman, 1971) and leprosy (Papageorgiou, Sorokin, Koutzouzakoglou & Glade, 1971), but IM remains the only condition in which there is a known temporal association between EB virus infection and the development of disease (Henle *et al.* 1968; Niederman, McCollum, Henle & Henle, 1968). It is also clear that although rising virus antibody titres have been observed in only a minority of patients with IM, virus antibody is always present in such patients. The disease does not develop in subjects with pre-existing EB virus antibody, and conversely only subjects lacking such antibody appear to be susceptible to the disease (Niederman *et al.* 1968; Evans, Niederman & McCollum, 1968).

In the past, understanding of IM has been hindered by ignorance of the nature and frequency of inapparent infection, and the presence of EB virus antibody now

provides the 'marker' of subclinical infection necessary for further epidemiological work. The majority of EB virus antibody studies so far reported have been made in student populations where IM is common, but there is little information about the epidemiology of apparent and inapparent EB virus infection in other groups. The aims of the present study were first, to seek evidence of recent EBV infection in patients with IM from the general population, and secondly to carry out a prospective study of the prevalance and spread of EBV infection among their contacts in the expectation that further light would be thrown on the aetiology and pathogenesis of IM.

PATIENTS AND METHODS

Index cases of IM were detected in three ways; admission to hospital, notifications by a group of general practitioners in the area served by St George's Hospital, S.W. 17, and requests to the hospital haematology laboratory for Paul–Bunnell tests. Index cases with a history and physical signs compatible with IM were admitted to the study if they developed a positive Paul–Bunnell–Davidsohn test or an atypical lymphocytosis. Five patients with neither of these abnormalities were also investigated as a result of highly suggestive clinical findings.

Patients and their families were visited as soon as possible after the onset of illness and clinical and epidemiological data recorded. Blood specimens were then taken from cases and contacts, and a record card was subsequently left on which the family were asked to record details of any illnesses occurring during follow up. Three months later, and in some cases at 6 and 9 months, the family was again visited, record cards collected and further specimens of blood taken from the index cases and contacts.

LABORATORY METHODS

Heterophile antibody

Patients' acute-stage sera were examined for heterophile antibody by a modification of a standard sheep-cell screening test (Brumfitt & O'Grady, 1957). The 'Monospot' slide test (Ortho Diagnostics), which detects heterophile antibody with the absorption pattern characteristic of IM, was also employed in the screening of both patients and contacts.

Sera giving a positive sheep-cell screening test or a positive 'Monospot' were tested by the full Paul–Bunnell–Davidsohn differential absorption procedure, using the method of Davidsohn & Henry (1968). This method uses doubling dilutions starting at 1/7. When sheep erythrocytes were used in this test, results were interpreted by the criteria of Davidsohn & Lee (1969). The test is positive provided that (i) the agglutinin titre after absorption with guinea-pig kidney is not more than eight-fold less than the unabsorbed titre and (ii) the titre after absorption with ox cells is at least sixteen-fold less than the unabsorbed titre. When the unabsorbed titre is 28 (all titres are expressed as reciprocals), any incomplete removal with guinea-pig kidney and complete removal with ox cells is interpreted as positive.

When horse erythrocytes were used the criteria for a positive test was that of Lee, Davidsohn & Slaby (1968), viz. that the titre after guinea-pig-kidney absorp-

tion should be higher than the titre after ox-cell absorption, irrespective of the unabsorbed titre.

The term 'Paul–Bunnell-positive' is used in this paper to denote a positive result in the Paul–Bunnell–Davidsohn differential absorption test using the criteria stated.

Atypical lymphocytes

Total and differential white cell counts were performed on all IM patients, and blood films were examined for atypical lymphocytosis. Blood films from family contacts were also so examined. Blood specimens were taken into EDTA tubes and films were prepared within six hours. The Jenner–Giemsa staining technique was used.

EBV antibody

Sera from index cases and contacts were examined for EBV antibody by an indirect immunofluorescence technique (Henle & Henle, 1966) using a modification of the method employed by the Virus Reference Laboratory, Central Public Health Laboratory, Colindale, London N.W. 9 (Pereira, Blake & Macrae, 1969).

The EB 3 line of Burkitt lymphoma cells was used as a source of antigen. Cells suspended in phosphate-buffered saline were transferred to antigen wells on glass microscope slides and air-dried. The preparations were fixed in acetone and stored at $-20°$ C.

For testing, sera were inactivated at $56°$ C. for 30 min. and then diluted, initially $1/8$ in phosphate-buffered saline. Doubling dilutions were then prepared using a Microtiter technique. A drop of each dilution was spread over each antigen well and the slides incubated at $37°$ C. for 1 hr. The slides were then washed thoroughly in three 10 min. changes of 0.9% saline and air-dried. A drop of fluorescein-labelled sheep anti-human immunoglobulin (Wellcome Reagents Ltd.) was spread over each antigen well and the slides incubated a further hour at $37°$ C. After the second incubation slides were washed in three 10 min. changes of phosphate-buffered saline (with mechanical agitation), air-dried, and mounted in phosphate-buffered glycerol.

The preparations were examined by ultraviolet microscopy. Known positive and negative control sera were included with each batch and the end-point read as the titre above which strongly fluorescent cells were no longer seen.

Cytomegalovirus, adenovirus and toxoplasma antibodies

Antibodies to these agents were measured in sera from all index cases. Complement-fixing antibody to cytomegalovirus was measured by the method of Stern & Elek (1965).

A standard method was used for the adenovirus complement-fixation test, using overnight fixation. Sera were examined for antibodies to *Toxoplasma gondii* by the dye test of Sabin & Feldman (1948). Sera from index cases with Paul–Bunnell negative IM which failed to show rising titres to EB virus, cytomegalovirus,

adenovirus or toxoplasma were tested for rubella haemagglutination-inhibition antibody (Stewart *et al.* 1967).

For all antibody measurements fourfold (or greater) rises or falls in titre were regarded as significant.

RESULTS

Patients with Paul–Bunnell positive IM

A total of 147 Paul–Bunnell positive patients was investigated. Their ages ranged from 3 to 48 years with a mean age of 18·6 years and a modal age of 16 years; 105 (71 %) were aged 14–23 years. Seventy-seven patients were male and 70 were female.

Epstein–Barr virus antibody results

All patients possessed EB virus antibody or developed it during or after illness. Antibody measurements were also carried out in a control group of 322 hospital surgical inpatients (without known IM, malignant disease, or other conditions known to be associated with high EBV antibody titres). Peak antibody titres from 100 IM patients were compared with the antibody titres in 100 age- and sex-matched controls. In contrast to the 100 % antibody prevalence in the IM group, only 78 % of the controls were antibody positive. However, the geometric mean titre of the patients' sera (73) was similar to that of sera from antibody-positive controls (76). High titres of antibody (equalling or exceeding 256) were also of similar frequency in both groups, being found in 11 % of patients and 14 % of antibody-positive controls.

At least two serum specimens were available from each patient. Of the 147 patients 93 (63 %) showed a significant change in antibody titre; 46 (31 %) with seroconversion, 44 (30 %) with a significant rise in titre and 3 (2 %) with a significant fall in titre. Although there was a wide scatter of results, seroconversion or a significant rise in titre was more likely when the first serum specimen was taken early in the illness. The first serum was taken within 10 days of onset in 41 of 90 patients (46 %) showing seroconversion, compared with 15 of 54 patients (28 %) whose antibody titres remained unchanged. Antibody was found in 69 % of sera taken during the first week of illness, 75 % of sera taken during the second and third weeks and 93 % of sera taken during the fourth and fifth weeks after onset. Detectable antibody was found in all sera taken after the fifth week. One patient was still EBV antibody negative on the 35th day after onset but developed a titre of 32 in a serum specimen taken on the 47th day after onset. A Paul–Bunnell–Davidsohn test was carried out for the first time on the 19th day and was positive.

Serial specimens were taken from twenty patients over a period of up to 16 weeks. The antibody titres of these patients in relation to the onset of illness are shown in Fig. 1. Titres rose until 6–8 weeks after onset; thereafter they showed no tendency to fall during the period of observation.

A pre-illness serum specimen was taken from five contacts who later developed IM, and fortuitously from two index cases. Of these seven patients six had no detectable EBV antibody before the onset of illness while the seventh had a low titre (8) in a serum specimen taken 45 days before onset.
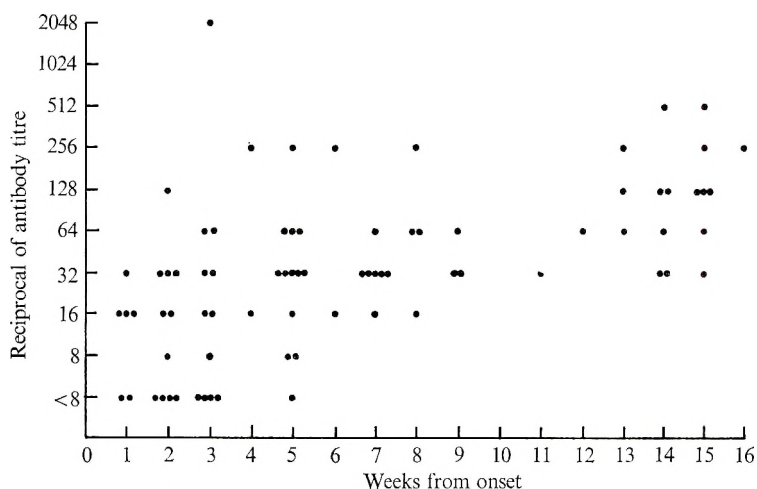
Fig. 1. Serial Epstein–Barr virus antibody measurements in twenty cases of infectious mononucleosis.

Toxoplasma antibody

Twenty-two of 146 patients (15%) had detectable antibody to *Toxoplasma gondii* at a titre of 16 or above. Of these, six had dye test titres of 512 or greater but in no case was there any significant change in titre.

Cytomegalovirus antibody

Three patients showed serological evidence of recent cytomegalovirus (CMV) infection during the first three months of follow-up. One of these acquired antibody to both CMV and EBV, a second showed a simultaneous rise in antibody to both these agents, and the third acquired antibody to CMV but had an unchanging EBV titre. This patient showed a strongly positive 'Monospot' test but there was insufficient serum for the Paul–Bunnell–Davidsohn titration.

Adenovirus antibody

Eighty-four of 137 patients (61%) had detectable adenovirus at a titre of 8 or above. Seventeen patients (12%) had a significant change in antibody titre; 8 with seroconversion, five with a significant rise and four with a significant fall.

Patients with Paul–Bunnell negative IM

Thirteen persistently Paul–Bunnell negative patients with 'glandular-fever-like' illnesses were studied; their ages ranged between 4 and 47 years.

Eight patients had an atypical lymphocytosis in the peripheral blood. Of these, three had serological evidence of recent EBV infection (two with rising titres and one with seroconversion) and two had serological evidence of recent cytomegalovirus infection (one with a rising titre and one with seroconversion).

Five patients had no atypical lymphocytosis; one of these showed a rising EBV antibody titre.

Of the thirteen patients with Paul–Bunnell negative IM, four showed serological evidence of recent EBV infection. Recent EBV infection may have been associated

Table 1. *Prevalence of EBV antibody*

Family contacts of Paul–Bunnell positive IM compared with controls

		Contacts					Controls		
Age	No.	No. pos.	Pos. (%)	Geometric mean titre of pos. sera	No. of sera with titres $\geqslant$ 1/256	No.	No. pos.	Pos. (%)	Geometric mean titre of pos. sera	No. of sera with titres $\geqslant$ 1/256
10–19	65	29	45	41	Nil	67	44	66	71	6
20–29	50	39	78	51	3	51	44	86	80	6
30–39	31	28	90	67	6	37	33	89	60	3
40–49	89	78	88	67	12	91	84	92	73	8
50–59	63	60	95	67	6	63	59	94	64	6
60–69	12	10	83	104	1	13	12	92	54	7
Totals										
10–19	65	29	45	41	Nil	67	44	66	71	6
20–69	245	215	88	NE*	28	255	232	91	NE	30
All ages	310	244	79	NE	28	322	276	86	NE	36

* NE = Not estimated.   Pos. = positive.

with the illnesses of five more patients but changing titres were not recorded. Cytomegalovirus mononucleosis was established in two patients, and in the remaining two patients, who had persistently negative EBV antibody titres, no aetiology was established.

Contacts of Paul–Bunnell positive patients

Prevalence of EBV Antibody

We investigated 335 contacts of 132 patients, of whom 265 (79 %) possessed EBV antibody. Antibody prevalence among *family* contacts in a number of age-groups was compared with that in controls (Table 1). A significant difference was observed only in the decade 10–19; 45 % of contacts in this age group (mostly patients' siblings) were antibody positive compared with 66 % of controls ($\chi^2 = 5\cdot9$; $0\cdot02 > P > 0\cdot01$). Antibody titres among contacts in this age group were lower than among the corresponding controls (mean ages 15·1 and 15·0 years respectively).

Recent EBV infection among contacts

Three hundred and six contacts were followed for periods of up to 9 months. Twelve of 63 contacts initially lacking EBV antibody underwent sero-conversion, seven during the first 13 weeks and five during the second 13 weeks of follow-up. In addition, nine antibody-positive contacts showed a significant change in antibody titre during the first 15 weeks of follow-up. The relationship between changing EBV antibody titres at different ages and the development of Paul–Bunnell positive IM, is shown in Table 2.

Heterophile antibody and atypical lymphocytosis

Initial and final sera from 218 contacts were examined by the 'Monospot' test. Seven specimens were 'Monospot' positive; five of these were taken in the acute

Table 2. *Recent EBV infection among contacts of Paul–Bunnell positive IM*

Age group	No. investi- gated	Contacts with recent EBV infection		No. developing Paul– Bunnell positive IM	Ages of contacts with recent EBV infection	
		No. lacking EBV anti- body at first visit	No. with changing EBV anti- body titre		Developing IM	Not developing IM
10–29	115	45	10, (7, 3)*	3	20, 21, 22	12, 13, 18, 18, 20, 22, 23
30–69	191	18	11, (5, 6)	2	31, 47	40, 42, 46, 52, 54, 58, 59, 61
Totals 10–69	306	63	21, (12, 9)	5	—	—

* The first figure in parentheses represents contacts with sero-conversion, the second represents those with a significant rise or fall in titre.

stage of illness from contacts who developed Paul–Bunnell positive IM. The final serum from a contact aged 20 with subclinical EBV antibody acquisition was also 'Monospot' positive; this serum gave a positive result in the Paul–Bunnell–Davidsohn test. Another positive was obtained on testing the initial serum from the wife of a patient with IM. This contact had had a sore throat three weeks before her husband's illness, but although she possessed EB virus antibody there was no serological evidence of recent infection.

Blood films were made from the initial and final specimens of 176 contacts. Apart from the secondary cases of IM already mentioned only one contact was found to have an atypical lymphocytosis. This was noted in the initial blood specimen from a 34-year-old female contact who was asymptomatic and had an unchanging EB virus antibody titre.

Contacts of Paul–Bunnell negative patients

Twenty-six contacts of the thirteen Paul–Bunnell negative patients were in-vestigated; in no case were significant clinical, serological or haematological changes observed.

Incubation period of IM

Seven patients developed Paul–Bunnell positive IM after a documented contact with the disease. Six of these secondary cases occurred in family members (four siblings and two spouses) who had been in continuous contact with a patient suffering from IM. In these instances the interval between the onset in the 'source' case and the onset in the 'secondary' case ranged from 6 to 23 weeks (mean; 14 weeks). The seventh patient became ill after intermittent contact with an IM patient for 8 weeks, the first contact occurring when the 'source' case had been ill for 6 days.

Fourteen other patients with Paul–Bunnell positive IM gave a history of possible contact but confirmation of the diagnosis in the 'source' case was only available in one instance.

DISCUSSION

This study confirms the constant association between Paul–Bunnell positive IM and the presence of EB virus antibody in a general population in Great Britain. Antibody was already present at first test or was shown to develop in all the patients, in contrast to an antibody prevalence of 78 % in controls. Evidence that EB virus was acquired at about the time of the illness was obtained in a considerably higher proportion of cases, 63 %, than in previous studies, since Niederman *et al.* (1968) found evidence of current infection in 7/29 of their patients and Joncas & Mitnyan (1970) showed a rising titre in only 15 of 129 cases. Although EB virus antibody usually appears early its late appearance has occasionally been described (Niederman *et al.* 1968). In this series 75 % of the patients had developed EBV antibody by the 3rd week of illness and all were positive after the 5th week.

The study of family contacts also demonstrated the association between susceptibility to IM and lack of EB virus antibody, since evidence of recent acquisition of virus was obtained in all five patients who developed the disease. Four of them acquired antibody *de novo* and one showed a rising titre over the period in which the disease became manifest.

Patients with clinical or haematological features of IM in whom the Paul–Bunnell (heterophile antibody) test is negative form an interesting group of diverse aetiology. Of thirteen patients who failed to develop heterophile antibody evidence of recent EBV infection was obtained in four, and of cytomegalovirus infection in two. Five of the thirteen patients showed no atypical mononucleosis and were included in the study because they had clinically typical glandular fever. It is notable that a rising EB virus antibody titre was shown in one patient who had neither heterophile antibody nor atypical lymphocytosis. Five of the thirteen patients showed no evidence of recent EB virus or of recent cytomegalovirus infection; they all possessed EB virus antibody, and some, of course, may have been infected shortly before the first serum specimen was obtained.

Epidemiological studies have shown that EB virus is acquired rapidly in all communities, and that the rate of acquisition is higher in poor than in rich communities (Porter, Wimberley & Benyesh-Melnick, 1969). Since frank infectious mononucleosis is rare both in childhood and in older adults it is of interest to note whether any particular syndromes could be associated with EB virus in age groups other than those liable to IM. Henle & Henle (1970) re-examined some of the sera from the Cleveland family survey, and found a great paucity of illnesses in these careful records which could have corresponded to acquisition of EB virus. It was possible, for example, to calculate that if EB virus were a cause of non-streptococcal pharyngitis, it could not have accounted for more than about 2 % of such illnesses. The susceptible contacts in our study, too, showed very little evidence of minor IM-like illnesses or haematological changes. Of twenty-one contacts with changing EBV antibody titres five developed Paul–Bunnell positive IM. Of the others,

eleven remained perfectly well during the 3–6 month period during which sero-conversion was detected and five had an episode of minor upper respiratory tract infection which could easily, of course, have been caused by one of many other agents. Haematological changes were not found in the contacts with any frequency, only one positive Paul–Bunnell–Davidsohn test being detected in a contact with subclinical EB virus infection, together with another positive 'Monospot' test in a contact with a history of recent sore throat who possessed EBV antibody at first test. Since these haematological changes are transient we might have found them more often with more frequent tests. Few of our contacts were young children, and the age of the infected contacts who acquired EB antibody without illness ranged from 12 to 69. It is notable that eleven patients over 40 showed evidence of recent infection although only one of them developed mononucleosis. Similarly, Wahren, Lantorp, Sterner & Espmark (1970) found four seroconversions among twelve parents in their family study. The relative immunity to IM of the older age groups cannot therefore be accounted for solely by the high prevalence of pre-existing antibody in this group.

The epidemiology of EB virus infection has been studied mainly in populations of young adults in which the disease is common. Indeed, the evidence suggesting that EB virus is a necessary cause of IM was obtained from a study of such a group in the U.S.A. (Niederman *et al.* 1968; Evans *et al.* 1968) and similar findings have recently been reported in Britain (University Health Physicians and P.H.L.S. Joint Investigation, 1971). Little is known of the epidemiology of IM in civilian populations and the immunofluorescent technique for the detection of antibody to EB virus has provided an epidemiological tool by which the spread of this agent may be traced. Wahren *et al.* (1970) noted change in antibody titre in nine of twenty family contacts and Joncas & Mitnyan (1970) demonstrated seven seroconversions among 67 antibody negative contacts of a group of 129 patients with IM. In our study 12 of 63 (19 %) of the initially negative contacts became infected during the 6 months after the index case was detected, while another nine contacts, already possessing antibody at first test, showed a change in titre. This rate of infection (or re-infection) among contacts suggests that virus is being excreted in the vicinity of the index case. Moreover, this estimate must represent a minimum value, since EBV antibody titres tend to level out soon after infection, so that we are unable to say how many of the group initially antibody positive had been recently infected. We hope to obtain further evidence on this point by other methods. This relatively high infectivity of the virus is not, of course, expressed clinically with any frequency, and only five contacts developed the disease. The secondary attack rate of IM was 6 % (4 of 63) among those not possessing pre-existing antibody, or 4 % (2 of 45) among the susceptibles aged 10–29 years. Yale students showed a remarkably high ratio of illness to inapparent infection of 2:1 (Niederman, Evans, Subrahmanyan & McCollum, 1970) while British students showed a ratio of 1:1 (University Health Physicians and P.H.L.S., 1971). In our contacts the overall ratio of illness to inapparent infection was approximately 1:3.

The evidence of causal association between EB virus infection and IM is based

on the insusceptibility to the disease in those already possessing antibody to the virus, and the finding that, conversely, all patients developing IM are derived from the group previously lacking antibody. The transmission of EB virus to cell cultures previously free from the virus has been claimed, using material from throat swabs or washings from patients with IM (Golden, Chang, Lou & Cooper, 1971; Pereira et al. 1972), but the problems of handling EB virus have made it difficult to prove unequivocally a causal association, and other interpretations of the association of EB virus with IM have been proffered (Hirshaut et al. 1969). One finding in this family study does provide support for the view that recent EB virus infection is a necessary cause of IM. The young family contacts, mostly sibs, of the index cases of IM showed a significantly lower prevalence of antibody than did their age-matched controls, suggesting that the cases of IM come from families with a lower than normal previous experience of this virus. For reasons already stated, moreover, the difference observed between two test and control groups is likely to be an underestimate. Antibody measurements in a normal community then tend to confirm the evidence of causal association between EB virus and IM, obtained from the study of student populations.

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The virulence of T-mycoplasmas, isolated from various animal species, assayed by intramammary inoculation in cattle

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SUMMARY

The virulence of T-mycoplasmas for cattle was tested by examining their ability to produce mastitis in cows. It was found that both virulent and avirulent strains of T-mycoplasmas can be isolated from cattle. All of four strains from pneumonic calf lungs and a strain from a case of bovine kerato-conjunctivitis caused mastitis but only two of four strains isolated from the urogenital tract of cows were virulent. None of the human, simian or canine T-mycoplasmas examined were able to cause mastitis in cattle. However, a bovine strain was found to be capable of causing mastitis in goats. Virulent and avirulent strains from the same and different species contain common antigens detected by the metabolic inhibition test. Pathogenicity could not be shown to be characteristic of any particular serotype. The possibility is raised of some species barrier being responsible for the inability of non-bovine strains to infect cattle.

INTRODUCTION

T-mycoplasmas have been isolated from the urogenital tract of cattle (Taylor-Robinson, Haig & Williams, 1967), pneumonic calf lungs (Gourlay, Mackenzie & Cooper, 1970) and from cases of bovine kerato-conjunctivitis (Gourlay & Thomas, 1969).

The virulence for cattle of two strains of T-mycoplasmas from pneumonic lesions of calves has been demonstrated by the findings that calves inoculated endo-bronchially developed pneumonia and cows inoculated via the teat canal developed experimental mastitis. However two human T-mycoplasma strains included in these tests did not cause mastitis in cows (Gourlay & Thomas, 1970; Gourlay, Howard & Brownlie, 1972).

An explanation for these findings could be that all human T-mycoplasmas are non-pathogenic. However, since both the bovine strains examined were isolated from the lung whereas the human strains came from the urogenital tract, pathogenicity might be related to the anatomical site of colonization. Furthermore, one of the cows challenged with a human strain (animal L91, Gourlay *et al.* 1972) appeared to be partially resistant to infection with the bovine strain and there may have been some variation in susceptibility among the experimental animals. Another explanation for the failure of human strains to cause mastitis in cows is that there may be an effective species barrier.

11-2

Table 1. *Strains used and their sources*

Strain no.	Source	
A417	Pneumonic calf lung	Compton
D32	Pneumonic calf lung	Compton
D20	Pneumonic calf lung	Compton
Vic9	Pneumonic calf lung	Compton
Bu2	Bovine urogenital tract	Compton
B101	Bovine urogenital tract	Compton
M525	Bovine urogenital tract	Compton
U12	Bovine urogenital tract	Compton
O13	Bovine eye	Compton
REOW	Human urogenital tract	Dr D. Taylor-Robinson
CD408	Human urogenital tract	Dr D. Taylor-Robinson
CD573	Human urogenital tract	Dr D. Taylor-Robinson
CD342	Human urogenital tract	Dr D. Taylor-Robinson
M126	Human urogenital tract	Dr B. E. Andrews
CD343	Human oral cavity	Dr D. Taylor-Robinson
Simian	Simian throat	Dr D. Taylor-Robinson
Canine	Canine urogenital tract	Dr D. Taylor-Robinson

The antigenic structure of sixteen of the seventeen strains of T-mycoplasmas used here has been reported previously (Howard & Gourlay, 1972). Strains were examined by a slight modification of the metabolic inhibition (MI) test of Purcell, Taylor-Robinson, Wong & Chanock (1966). The strains were serologically heterogeneous and the possibility existed that virulence might be a characteristic of particular serotypes. The virulence of strains of T-mycoplasmas from various anatomical sites and species was studied by intramammary inoculation of cattle to answer some of the specific questions raised concerning the pathogenicity of T-mycoplasmas.

MATERIALS AND METHODS

T-mycoplasma strains

Strains A417 and D32 were isolated from pneumonic calf lungs and have been described previously (Gourlay *et al.* 1972). Strains Vic9 and D20 were also isolated from the lungs of calves with pneumonia. O13 was isolated from the eye of a cow with kerato-conjunctivitis (Gourlay & Thomas, 1969). Strains Bu2, M525, U12 and B101 were all isolated from the urogenital tract of cows. The bovine strains were purified as previously described (Gourlay *et al.* 1972). All the other strains were obtained from Dr D. Taylor-Robinson except strain M126 (Table 1). The canine and simian strains as well as strains CD343 (Johnson) and REOW have been described by Taylor-Robinson, Martin-Bourgon, Watanabe & Addey (1971). The human strains were isolated from the urogenital tract except strain CD343 which originated from the oral cavity. The strains and their sources are listed in Table 1.

All strains were grown in U-broth without Hepes (Gourlay *et al.* 1972).

Inoculation of animals

Cows and goats in milk were inoculated via the teat canal with 10 ml of actively growing mycoplasma cultures. The number of T-mycoplasmas and the number of
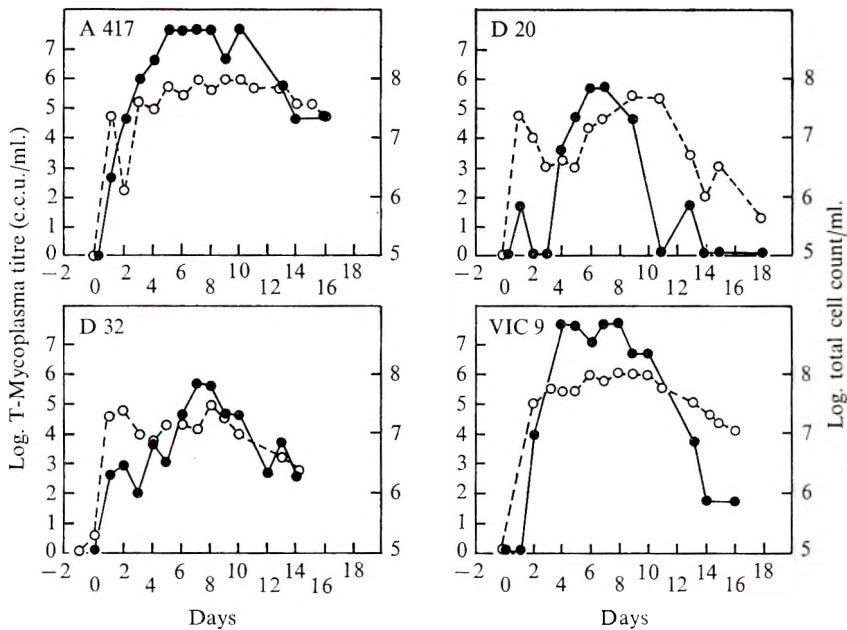
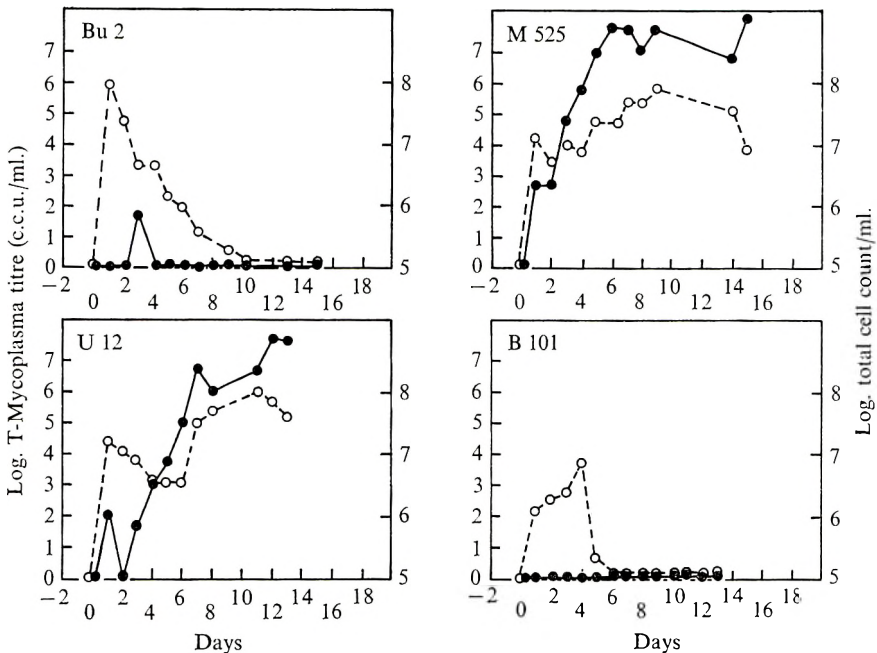Fig. 1. Number of T-mycoplasmas and cells in the milk of cows inoculated with four strains from pneumonic calf lungs. Inocula: A417, $10^{5.7}$; D20, $10^{5.7}$; D32, $10^{5.7}$ and Vic9, $10^{5.7}$ c.c.u./ml. ●—● T-mycoplasmas; ○--○ cells.

cells present in milk were measured as previously described (Gourlay *et al.* 1972) except that the T-mycoplasma titre was recorded as the 50 % endpoint (Gourlay & Domermuth, 1967). The criteria used to determine whether strains produced mastitis following intramammary inoculation were the continued increase in the number of cells in milk associated with the consistent reisolation of T-mycoplasmas from milk. Strains which did not cause infection were tested in at least two animals. Each animal was inoculated at the same time in another quarter with strain A417 as a control. The possibility of a concurrent bacterial mastitis occurring was excluded by spreading blood agar plates with milk and examining them for bacterial colonies.

<center>RESULTS</center>

Strains from calf lungs

Of 20 cows inoculated with strain A417, 19 developed mastitis. In one case the animal appeared to be partially immune to infection (animal L91, Gourlay *et al.* 1972). Another cow was found to be refractory to infection with strain A417, although it was susceptible to infection with the bovine urogenital strain U12. A typical response to strain A417 is shown in Fig. 1. The maximum T-mycoplasma titre and number of cells in the milk occurred about 5–10 days after injection. Mycoplasmas have been found to be excreted for as long as 6 months after inoculation, the longest time studied. The lowest dose of actively growing T-mycoplasmas that has been inoculated was 10^4/ml. strain A417, and this caused mastitis.

The response of cows to inoculation with three other T-mycoplasma strains isolated from cases of calf pneumonia is shown in Fig. 1. All of these strains caused

Fig. 2. Number of T-mycoplasmas and cells in the milk of cows inoculated with four strains from the urogenital tract of cows. Inocula: Bu2, $10^{6.7}$; M525, $10^{6.7}$; U12, 10^7 and B101, $10^{6.7}$ c.c.u./ml. ●——● T-mycoplasmas; ○--○ cells.

mastitis, but they varied in their ability to persist and multiply in the udder. Infection with strain D20 was resolved rapidly compared to infection with A417. Infection with all of these strains, except D20, caused the milk to become yellow and produced clots and could thus be considered to have caused clinical mastitis. Strain D20 caused only subclinical mastitis.

Strains from the urogenital tract of cows

Four strains isolated from the urogenital tract of cattle were tested for virulence. Two of these four strains, U12 and M525, proved virulent on intramammary inoculation and caused the milk to become yellow and produced clots. The number of milk cells and T-mycoplasmas found in the milk after inoculation is shown in Fig. 2. The infection was as severe as that caused by strains isolated from the lung. Two of the urogenital strains examined, B101 and Bu2, were avirulent, the response to their injection was essentially similar to that produced by non-viable cells of strain A417 (Gourlay *et al.* 1972). Inoculation of B101 and Bu2 caused a transient cell response in the milk (Fig. 2).

A T-mycoplasma strain from the eye of a cow

The results of inoculation with strain O13 are shown in Fig. 3. This strain caused clinical mastitis as evidenced by the yellow milk produced subsequent to injection.

T-mycoplasmas isolated from man

Six human T-mycoplasmas listed in table 1 have been tested for their ability to cause mastitis in cows. Five of the strains were from the urogenital tract and one
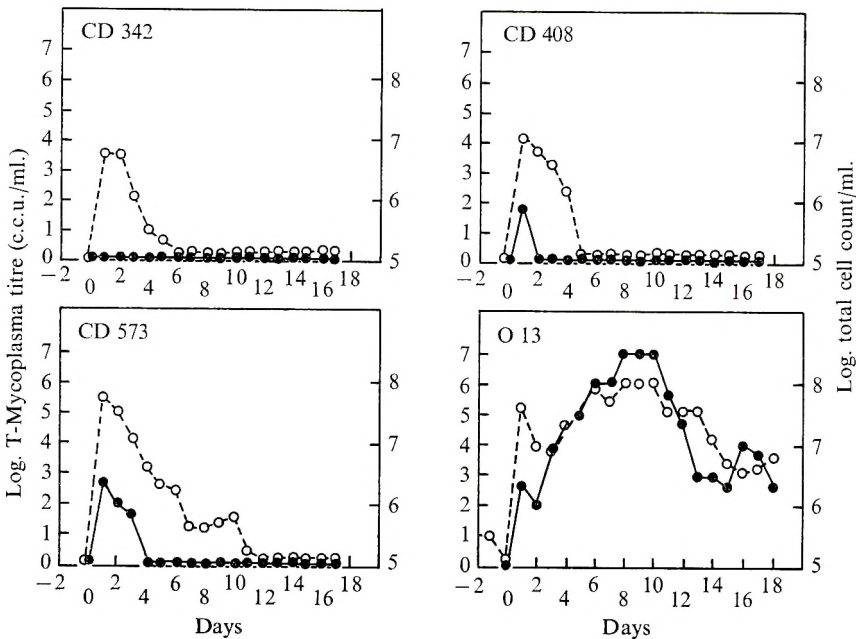
Fig. 3. Number of T-mycoplasmas and cells in the milk of cows inoculated with three human strains and a strain (O13) from the eye of a cow. Inocula: CD342, $10^{6 \cdot 7}$; CD408, 10^{6}; CD573, $10^{6 \cdot 7}$ and O13, $10^{5 \cdot 7}$ c.c.u./ml. ●—● T-mycoplasmas; ○--○ cells.

was from the oral cavity (CD343). All the strains were inoculated into at least two different cows. None of the human strains was virulent for cattle. The response induced by three of the human T-mycoplasmas is shown in Fig. 3. In all cases on the day after injection a cell response was found in the milk but the high level of cells did not persist. T-mycoplasmas were sometimes isolated on the first or even second and third day after inoculation but this was considered to be due to the persistence of the inoculum. No gross milk changes or udder abnormalities were observed following inoculation with human T-mycoplasmas. The response of cows to human strains was essentially the same as that produced by inactivated bovine T-mycoplasma strain A417 (Gourlay *et al.* 1972) and by the avirulent bovine strains.

Simian and canine T-mycoplasmas

Neither of these strains caused mastitis in cows. The type of response produced by their inoculation was identical with that of human T-mycoplasmas and the avirulent bovine strains. Neither of the strains multiplied in the udder and they were not re-isolated from the milk. The cell response induced was maximal on day one after injection and thereafter the number of leucocytes in the milk gradually declined. No gross milk or udder abnormalities were observed.

Infection of goats with bovine T-mycoplasma

Goats were challenged in the same way as cows with strain A417. An infection with clinical signs of mastitis was produced in four out of four animals. An increase
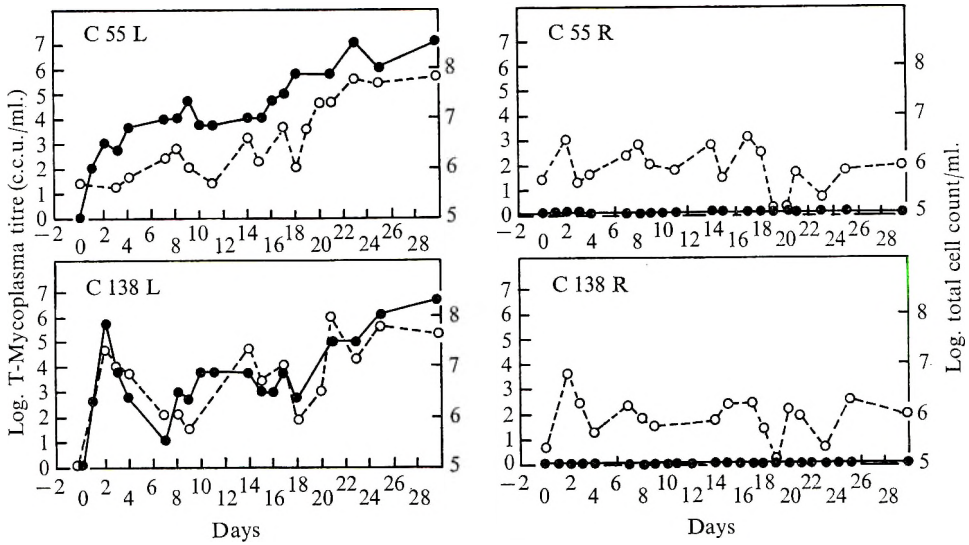
Fig. 4. Number of T-mycoplasmas and cells found in the milk of two goats, nos. C55 and C138, inoculated with bovine strain A417. Inocula: left (L) mammary gland A417, $10^{4\cdot7}$ c.c.u./ml.; right (R) mammary gland U-broth. ●—● T-mycoplasmas; ○--○ cells.

in the number of leucocytes in the milk was observed and T-mycoplasmas were reisolated from the milk samples (Fig. 4). The number of cells found in control glands injected with U-broth was higher than in cows. The infections caused an increase in the number of cells present and gross milk changes were apparent.

DISCUSSION

All four T-mycoplasmas isolated from pneumonic calf lungs and the strain from a case of bovine kerato-conjunctivitis were found to be virulent for cattle. However, both virulent and avirulent T-mycoplasmas have been isolated from the urogenital tract of cows. Pathogenicity is not therefore a specific feature of bovine strains isolated from a particular site.

It is possible that the urogenital tract of cows acts as a reservoir of T-mycoplasmas. The upper respiratory tract could become infected as calves pass down the birth canal. Klein, Buckland & Finland (1969) considered that the oral cavity of babies can become infected with T-mycoplasmas during parturition.

Although T-mycoplasmas have been incriminated in urogenital tract infections of man, there is still doubt regarding their role in these conditions (Shepard, 1969; Taylor-Robinson, 1971; Ford, 1970) and it has been suggested that T-mycoplasmas may usually be commensals in man (Klein *et al.* 1969; Biberfeld, 1971) and in the urogenital tract of bulls (Taylor-Robinson, Thomas & Dawson, 1969).

The possibility that T-mycoplasmas may be of aetiological significance in calf pneumonia has been suggested by their isolation from 58 % of pneumonic calf lungs (Gourlay *et al.* 1970) but not from non-pneumonic lungs (Thomas & Smith, 1972). Furthermore, they cause pneumonia in calves inoculated endobronchially

(Gourlay & Thomas, 1970). The finding that all four strains isolated from calf pneumonia were virulent, unlike the bovine urogenital strains, is consistent with the possibility that T-mycoplasmas are of aetiological significance in calf pneumonia. This finding may be the result of a selective pressure being present in the respiratory tract which is not present in the urogenital tract.

Taylor-Robinson (1971) reported that the inoculation of a bovine T-mycoplasma into the urethra of a Caesarian-derived pathogen-free bull-calf caused infection but failed to produce disease. However, bovine T-mycoplasmas are capable of causing clinical mastitis in cows infected experimentally. This group of micro-organisms should not be regarded as merely commensals in cattle.

None of the human, canine or simian strains tested caused infection in cows. An explanation for this finding could be that all the strains examined were avirulent. Since both virulent and avirulent T-mycoplasmas have been isolated from cattle there is by analogy no reason for assuming that all human, simian and canine strains are avirulent *per se* and an alternative explanation is that some host specific factors are involved which prevent the non-bovine strains from infecting cows. However, since the bovine A417 strain was capable of causing experimental mastitis in goats, host specificity is not absolute, although specific strains may only be able to infect a limited range of animals. The findings reported by Taylor-Robinson *et al.* (1971) that only human T-mycoplasmas adsorbed to HeLa cells, not bovine, simian or canine strains, and only simian strains adsorbed to chicken erythrocytes indicates that some specificity exists in cell adsorption by T-mycoplasmas.

Human and bovine T-mycoplasmas have been found to be serologically heterogeneous. Strains which contain common antigens can be isolated from various anatomical sites and from normal or diseased conditions (Ford, 1967; Purcell, Chanock & Taylor-Robinson, 1969; Taylor-Robinson *et al.* 1969; Howard & Gourlay, 1972). Furthermore, strains from different animal species have been found to cross react in the MI test (Howard & Gourlay, 1972).

Since strains that had been reisolated from milk reacted with the antisera in the same way as they did before injection, the antigenic structure of the organisms is apparently a stable characteristic.

Sixteen of the strains tested for virulence have been examined by the MI test for cross-reacting antigens using antisera raised against six of the strains (Howard & Gourlay, 1972).

Both virulent and avirulent bovine T-mycoplasmas possess common antigens. Moreover, strains from other species which contain antigens present in virulent bovine T-mycoplasmas are avirulent for cows. Judging from the results presented here, no particular serotype, as indicated by the MI test, appears to be characteristically pathogenic.

The results reported by Taylor-Robinson *et al.* (1971), noted above, indicated differences in the cell adsorptive properties of strains from different species. Our results indicate that important differences between strains from the same and different animal species exist which affect the virulence of strains for a particular species of animal.

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Studies on transmission of *Staphylococcus aureus* in an isolation ward for burned patients

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SUMMARY

A one-year epidemiological investigation was made in an isolation ward for burned patients. The transmission of *Staphylococcus aureus* was mainly studied. In spite of the design of the ward the cross-infection rate was high. In all, 49 of 69 patients were infected 114 times. Twenty-six of the strains causing infection were found in a patient only, 10 in a member of the staff only and 23 in both patients and staff the week before they caused a new infection. There were three epidemic outbreaks caused by three strains of *Staph. aureus* all belonging to phage group III; one was resistant to methicillin. Environmental studies with settle plates showed that the number of staphylococci dispersed by a burned patient was often very high. In 8 % of the observations in occupied bedrooms the air count of *Staph. aureus* was more than 1800 col./m.² hr. However, the counts of *Staph. aureus* in the corridor and service areas were low. This seems to indicate a rather good protection against airborne transfer of bacteria. Other routes of infection were probably of greater importance.

INTRODUCTION

Modern medicine has made it possible to treat extensive burns with good results.

Infections are, however, still very common and the dominant bacteria are staphylococci, beta haemolytic streptococci and gram-negative bacteria such as *Pseudomonas aeruginosa* and coliforms.

Several methods of preventing infections have been tried. Amongst these are a number of different kinds of local treatment which have had varying success (Cason, Jackson, Lowbury & Ricketts, 1966; Weyer, Krauss & Sussell, 1968; Matter, Barclay & Koníčková, 1970). Isolation is generally regarded as an important measure for the protection of those patients highly susceptible to infection and the usual method of providing this is in single-bedded rooms, preferably with positive-pressure mechanical ventilation. The main object of this system is to reduce the risk of airborne transfer of infection from one room to another, but doubtless the risk of transfer through fomites and contact is also reduced.

In October 1968 an isolation ward for burned patients was opened at the University Hospital in Uppsala. The effectiveness of the isolation provided in this ward has been studied from different aspects and the results will be presented in a series of papers. These studies have mainly been concerned with the behaviour
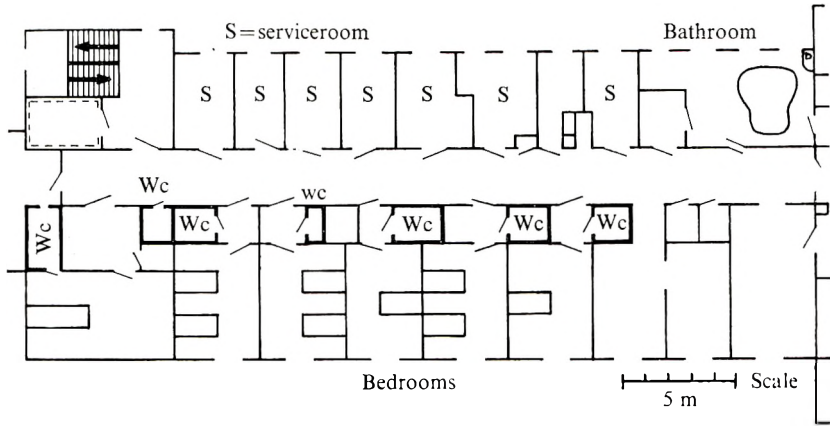
Fig. 1. Plan of the burns ward.

of *Staph. aureus* as it is evident that these bacteria are conveyed between patients by air as well as by contact (Lowbury, Babb & Ford, 1971).

In this paper the epidemiological events during the first year the ward has been in use will be reported. The results of investigations into the transmission of *Staph. aureus* by airborne routes and by means of nurses' gowns and protective clothing will be published separately.

MATERIALS AND METHODS

Isolation ward

Figure 1 shows the plan of the isolation ward. It is entered via an airlock with double doors, and a corridor runs down the middle of the ward. There are five bedrooms of similar dimensions and a sixth larger room containing an airbed. All of these have individual airlocks and are situated along one side of the corridor. On the opposite side of the corridor are service rooms and a bathroom. The bathroom has three doorways: one of these opens directly to the corridor for bed transport and the other two open via airlocks, one into the ward entrance airlock and the other into the corridor. The ventilation rate is approximately 4 air changes per hour in the patient rooms (Hambraeus & Sanderson, 1972).

Clinical material

The primary investigation period lasted from October 1968 to October 1969 and covered 52 weeks. During this time 80 patients were treated in the unit. Of these, 49 patients had small burns, 22 medium sized, and 9 extensive burns. The extent of burns and age of the patients is shown in Table 1.

Length of stay in the ward for about half the patients was less than 3 weeks, one third stayed between 4 to 7 weeks, and ten patients stayed for more than 8 weeks. Seven patients, all with extensive burns, died.

The method of treatment was exposure and bathing. After 2 to 3 weeks exposure the patients were bathed 2–3 times per week and necrotic tissue was excised at the same time.

Table 1. *Distribution of the material according to age of patient and size of burn*

Age in years	Small burns < 15 % totally < 5 % III°*	Medium-sized burns 15–30 %totally 5–15 % III° *	Extensive burns > 30 % totally > 15 % III° *	Total
0–5	14	6	—	20
6–20	2	4	—	6
21–50	17	7	7	31
> 50	16	5	2	23
Total	49	22	9	80

* III° = third degree.

From each patient specimens were taken from nose, throat, wound and perineum on admission and thereafter once a week or if necessary more often. The total numbers of specimens were 354 from wounds, 349 from nose and throat, 331 from skin and 330 from perineum.

Staff

About 200 persons worked in the ward during the investigation period. Of these 36 belonged to the more permanent staff and 11 were doctors and research staff. Specimens from nose and throat were taken from the staff twice before the ward was opened and thereafter once a week. The number of nose and throat specimens was 974.

Antibiotic policy

Prophylactic antibiotic therapy with penicillin was used for moderate and severe wounds. In cases of clinical infections antibiotic therapy was directed by sensitivity determinations of isolated bacteria.

Routine

Protective gowns and masks were used when treating the patients in their rooms. The gowns were kept in the airlock and changed once a day. For newly admitted uninfected patients sterile gowns, gloves, caps and masks were used. The bathtub was cleaned with a phenolic compound (0·5 % Gevisol).

Bacteriological technique

Swabs from nose, throat, skin and perineum were plated on blood and phenol-mannitol-agar. For specimens from clinical infections blood-agar with gentian violet, haematin and anaerobic blood-agar were also used. After incubation at 37° C. for 24 and 48 hr. the plates were read and colonies picked for identification and typing. Presumptive *Staphylococcus aureus* colonies were tested for deoxy-ribonuclease production. Positive strains were phage typed with the international set of phages (Blair & Williams, 1961). All strains from wound specimens and to some extent from other specimens were tested for antibiotic resistance according to Ericsson's disk diffusion method (Ericsson, Högman & Wickman, 1954). Although the main purpose of the investigation was to study the epidemiology of

Table 2. *Analysis of bacteriological findings from 354 wound cultures in 76 patients*

Organism isolated	Relative frequency in culture (%)			Relative frequency in patients (%)		
	Pure	Mixed	Total	Sole organism isolated	Mixed infection	Total
Staph. aureus	37·6	29·1	66·7	12	66	78
Staph. albus	7·1	10·5	17·6	4	47	51
Coliforms	0·3	20·1	20·4	—	40	40
Pseudomonas spp.	0·6	5·4	6·0	1	12	13
Proteus spp.	—	5·1	5·1	—	7	7
Enterococci	0·3	8·2	8·5	—	24	24
β-streptococcus (group A)	1·7	0·6	2·3	—	7	7
β-streptococcus (other than group A)	—	2·3	2·3	—	9	9

Staph. aureus, isolation and typing of haemolytic streptococci and *Ps. aeruginosa* was also carried out. In specimens from clinical infections all potentially pathogenic organisms were isolated and identified according to current methods.

Environmental studies

For this purpose settle plates were used and they contained 2 μg. nalidixic acid per ml. to prevent swarming of proteus. They were placed at 8 fixed places in the corridor, service rooms and bathroom and after the 19th week of the investigation also in the bedrooms. They were exposed for 4 hours 3–5 days a week and then examined for presence of *Staph. aureus* as described above. All *Staph. aureus* colonies found on settle plates in the corridor and service rooms, together with 25 % of colonies (up to a maximum of 8 per plate) from settle plates in the bedrooms, were phage typed. In all, 1163 settle plates were examined from corridor and service rooms, 165 from the bathroom and 456 from bedrooms. Swabbings from three different sites in the bathtub have been made 90 times in all, i.e. about twice a week.

Bacteriological findings

Patients. Wound specimens from 76 of the 80 patients admitted to the ward, 354 in all, were examined. *Staph. aureus* was the most common organism found. The occurrence of different pathogens in the wound specimens is shown in Table 2. In 59 patients *Staph. aureus* was isolated, and in 9 of these patients it was the only pathogenic organism isolated from the wound during the stay in the ward. As the main purpose of this investigation was to study the epidemiology of *Staph. aureus* these are the only organisms to be reported in detail.

RESULTS

Specimens from wound, upper respiratory tract, skin and perineum were taken from 76 patients on the day of admission. In 35 of these patients no *Staph. aureus* could be isolated. In 18 patients *Staph. aureus* was isolated from the wound and

other sites, in 5 patients from the wound only, and in 18 patients only from sites other than the wound, usually the upper respiratory tract. In all, 69 patients were examined on two or more occasions and 49 of these were shown to have acquired hospital strains of *Staph. aureus*. In 5 of these cases *Staph. aureus* could never be isolated from the wound but only from the upper respiratory tract, skin or perineum.

Thirty-six patients acquired a new strain of *Staph. aureus* during the first week of their stay in the ward, 9 during the second or third week and 4 patients between the third and fourth week. No less than 26 patients became infected before the isolation had been broken and the patients had been taken out of their rooms for bathing or operation.

Of the 20 patients who did not become infected during their stay, 8 already had *Staph. aureus* in their wounds on admission and the others were treated for less than three weeks.

It was not uncommon for patients to become infected with *Staph. aureus* of more than one phage type. Fourteen patients were infected with two strains, and 8 with three strains, 7 with four strains and 3 with five. As could be expected the risk of acquiring new strains increased with length of stay in the ward. Of the 10 patients infected with 4 or more types only 2 stayed for less than 6 weeks and they both had extensive burns.

Staff

An analysis of the carrier situation was made in 39 people who worked in the ward for more than six weeks. *Staph. aureus* was found in 80 % or more of consecutive cultures from the upper respiratory tract in 18 persons, in 45 to 80 % in 14, and in less than 45 % in 7. The number of strains acquired per person varied from 1 to 8, the mean value being 2. Two members of the staff had wounds infected with hospital strains.

Phage typing

A total of 1827 *Staph. aureus* isolates were phage typed, and amongst these 155 patterns could be recognized. Table 3 shows the distribution of phage groups among patients and staff, the lytic pattern of 11 strains isolated from more than 2 patients is also presented. Group III strains were the most common among patients and staff, the dominating phage types being type 84, 53/77/84 and 88 typable at 1000 RTD only, which were isolated from 54 % of the patients and 20 % of the staff. As might be expected types belonging to 52/52A/80/81 complex were fairly common too, these were isolated from about 30 % of both patients and staff.

Environmental studies

Table 4 shows the results of the environmental studies with settle plates. The number of *Staph. aureus* colonies isolated on plates exposed in occupied bedrooms varied considerably: 27 % of the exposed plates yielded no colonies, 31 % had 1–5 colonies, 8 % had more than 100 (i.e. about 1800 col./m.^{2} hr.) the highest

Table 3. *Occurrence of various phage-types/groups in specimens from patients and staff*

Phage-type/group	No. of patients	No. of staff
52/52A/80/81 complex	23	57
Rest of group I	5	12
Total of group I	28	69
55/71	3	2
3A/55 × 1000 RTD	3	3
Rest of group II	7	24
Total of group II	13	29
84	24	34
53/77/84	10	7
88 × 1000 RTD	7	8
6/47/54/85	4	3
54 × 1000 RTD	4	3
Rest of group III	24	58
Total of group III	73	113
Total of group IV	1	1
187	3	1
52A/55/6/53/54 × 1000 RTD	4	2
52/52A/42E/54 × 1000 RTD	3	5
Rest of mixed group	11	21
Total of mixed group	18	28
N.T.	27	44

N.T. = Not typed.

Table 4. *Distribution of colony-forming units of* Staph. aureus *on settle plates in corridor, service rooms, bedrooms and bathroom*

	Number of settle plates		
C.f.u. *Staph. aureus* settle plate	Bedrooms	Corridor and service rooms	Bathroom
0	122 (26·8 %)	705 (60·6 %)	75 (45·6 %)
1–5	141 (30·9 %)	420 (36·1 %)	38 (23·0 %)
6–10	52 (11·4 %)	28 (2·4 %)	21 (12·7 %)
11–20	35 (7·7 %)	9 (0·8 %)	11 (6·7 %)
21–50	45 (9·9 %)	1 (0·1 %)	11 (6·7 %)
51–100	23 (5·0 %)	—	7 (4·2 %)
101–200	19 (4·2 %)	—	2 (1·2 %)
201–400	10 (2·2 %)	—	—
> 400	9 (2·0 %)	—	—
Total	456	1163	165

count being about 800. In the corridor and service areas the variation was less and the counts lower than in the bedrooms: 61 % yielded no colonies of *Staph. aureus*, 0·9 % had more than 10 colonies and no plate yielded more than 30 colonies (i.e. 540 col./m.² hr.). The number of staphylococci found in the bathroom was often rather high; 12 % of the plates had more than 20 col.
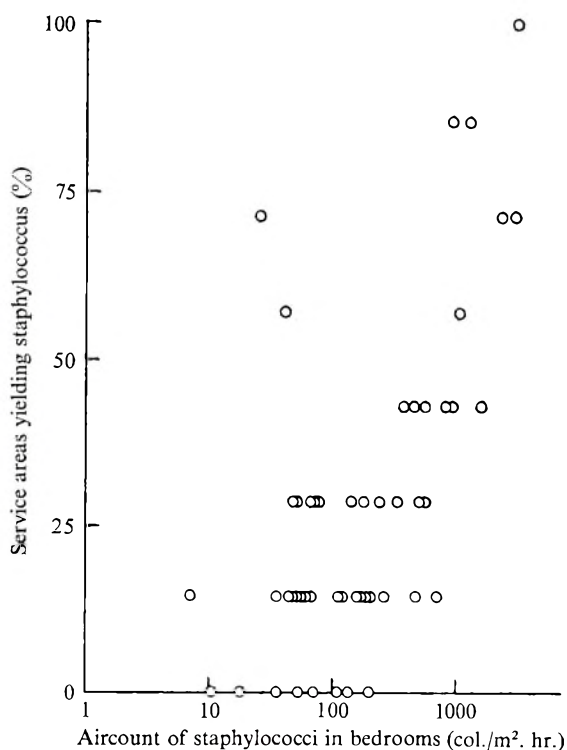
Fig. 2. Spread of staphylococci from bedrooms to service areas.

In Fig. 2 the mean number of *Staph. aureus* found/m.^{2} hr. in the bedrooms is plotted against percentage of service areas yielding staphylococcus. The presence of staphylococci is given without regard to differences in phage types. As transport of patients in the passage and the presence of a disperser in the bathroom causes a break in the isolation system those occasions when staphylococci were found in the bathroom have been excluded.

It is seen in Fig. 2 that on only 8 occasions (16% of the total) were staphylococci found on more than half the plates exposed in the passage and service areas. When, however, the mean count in the bedrooms exceeded 1000 colonies/m.^{2} hr., then staphylococci were recovered from more than half the plates from passage and service areas on 75% of occasions (6/8).

Sixteen of the 90 specimens from the bathtub yielded small amounts of staphylococci, they were usually found on the immersion lift used to lower the patient into the water.

Epidemiological results

The epidemiological situation in the ward was complicated. In all, 114 strains colonized 49 patients. Twenty-six of these strains were found in a patient, 10 in a member of the staff and 23 in both patients and staff the week before it caused a new infection. Sixteen of the strains were isolated from several persons in the ward in the same week thus making it impossible to deduce who infected whom. In 39 cases no source of infection was found in the ward.
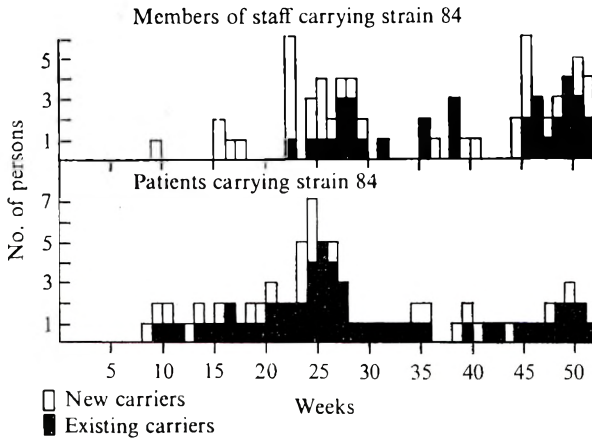
Fig. 3. Course of the epidemic due to *Staph. aureus* phage type 84 III.

As seen in table 3 11 phage types were isolated from more than 2 patients. Although types belonging to the 52/52A/80/81 complex were isolated from 23 of the patients and 57 members of the staff, it was only possible to show epidemiological relationship with any certainty in 8 of these persons, 3 patients and 5 members of the staff. Thus there remain only 3 types of *Staph. aureus* that caused epidemic outbreaks. Some aspects of these epidemics are now described.

Strain phage type 84

Strains of this phage type were easily identified. They were interesting in that they were multiresistant even to methicillin and produced enterotoxin B. The strain was first isolated from a settle plate in week 8; its origin could not be found. The week after, *Staph. aureus* phage type 84 was isolated from a patient with a small burn wound. He was admitted from another ward of the hospital and the first strain isolated from his wound had another phage pattern.

The course of the epidemic can be seen in Fig. 3. Up to week 21 there were only sporadic cases, 8 altogether. Four of these 8 patients were probably infected because they shared rooms with carriers. During weeks 22 and 23 no new case occurred. During weeks 24 to 27 there was an epidemic outbreak with 7 new cases. In fact all patients treated in the ward during weeks 24 and 25 were infected with the strain. The last 8 cases occurred sporadically during the following 26 weeks. In all, the strain was isolated from 24 patients, and in 20 of them from the burn as well as from the upper respiratory tract, skin or perineum. In 4 cases the strain could not be isolated from the burn but only from other sites. In 16 cases the infection occurred after the isolation had been broken and the patients were brought out of their rooms for bathing or operation. The operating room was not a very likely source of infection since it was also used for other surgical specialities where no infection occurred with this strain. The bathtub in the bathroom was a more likely source. In 9 of the 16 cases where *Staph. aureus* was isolated from the bathtub the isolated strain was of phage type 84. It is, however, difficult to determine the possible mode of acquisition in any one case; of the 3 patients infected during
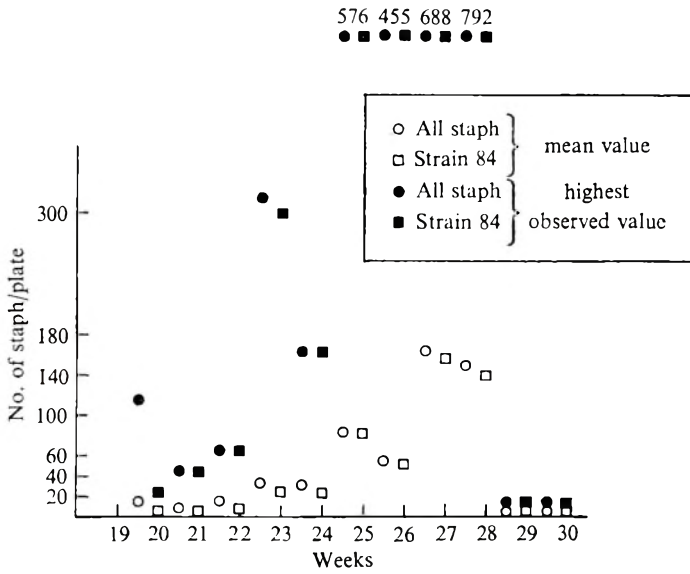
576 455 688 792

● ■ ● ■ ● ■ ● ■

Fig. 4. Weekly settle plate counts of *Staph. aureus* phage type 84 III.
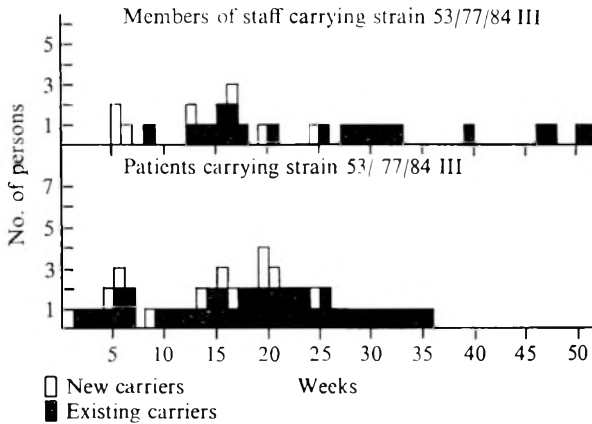

Fig. 5. Course of the epidemic due to *Staph. aureus* phage type 53/77/84 III.

week 24 2 had been operated upon and 1 had been bathed; of the 3 patients infected during week 25, two shared a bedroom, and neither had been bathed or operated upon.

With regard to the possibility of airborne infection it can be seen from Fig. 4 that the week before the epidemic outbreak among the patients there was an increase in the number of colonies with phage type 84 found on settle plates in the bedrooms. This was due to one of the patients becoming a heavy disperser. The same patient caused the high values found during weeks 23 to 25; thereafter two other extensively burned patients began to disperse bacteria. During these weeks there was probably transfer of *Staph. aureus* through the airlock out into the passage, and an increase of staphylococcus found on settle plates in the corridor was noticed.

12-2
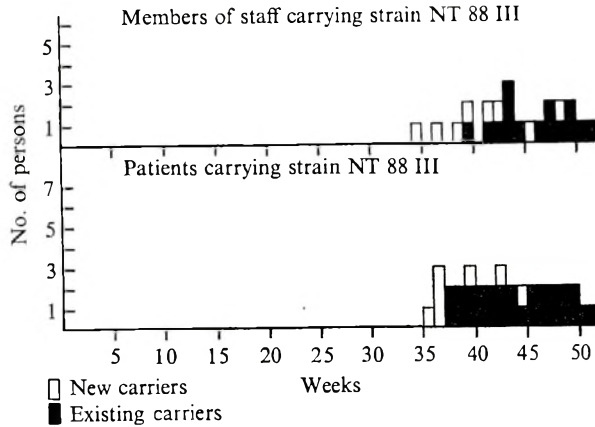
Fig. 6. Course of the epidemic due to *Staph. aureus* phage type 88 III.

Figure 3 shows that phage type 84 was first isolated from the staff after it had been found among the patients. Of interest, however, is the large number of carriers observed during week 23, when the strain was isolated from 6 members of the staff. This high carrier-rate preceded the outbreak among the patients, and after this cumulation which continued up to week 30 the number of carriers decreased again. From week 44 and thereafter was a new increase of carriers and the strain was found among 11 members of the staff, one of whom had a clinical hand infection. This new increase of carriers, however, was not followed by any new outbreak of infections among the patients.

Strain phage type 53/77/84

This was also a multiresistant hospital strain producing enterotoxin B. It was, however, sensitive to chloramphenicol and methicillin. It was brought into the ward during the first week by a patient who was admitted to the ward from another hospital. The course of this smaller epidemic in which ten patients became infected can be seen in Fig. 5. The first infection in the ward occurred during week 5 in a patient who shared a room with the one who introduced the strain into the ward. The remaining 9 cases occurred sporadically during the first 25 weeks the unit was in use and there was no accumulation at any specific period.

In all cases this strain could be isolated from the burn as well as from the upper respiratory tract, skin or perineum. Of epidemiological interest is that before the infection occurred, isolation had been broken for 4 patients because of operation and bathing, and for another 2 for operation.

The strain was isolated from 2 members of staff during the 6th week and thereafter from another 5 between weeks 7 and 25. After that 1 member of the staff was a carrier of the strain for the rest of the year, and there was 1 case of wound infection among the staff.

It is difficult to draw any definite conclusions from environmental studies since settle plates were not used in the bedrooms until after week 19. *Staph. aureus* of type 53/77/84 were, however, only sporadically found on the settle plates in the

corridor and service rooms during these first 19 weeks so that it is not very likely that there were any heavy dispersers among patients treated in the ward unit during this period. After week 19 strains of this type were found on settle plates in the bedrooms but usually only in small numbers.

Strain phage type 88

This strain was resistant to penicillin but sensitive to tetracycline, chloramphenicol and methicillin. It was typable only at 1000 RTD and was first isolated from the upper respiratory tract of a nurse during week 35. The course of the epidemic in which 7 patients became infected can be seen in Fig. 6. The first case occurred during week 36, one week after it was first found among the staff. The week after there was a small accumulation with 3 new cases and during the following 8 weeks there were another 3 isolated cases. All these patients had been treated in different rooms, but in all cases the isolation had been broken before the infection occurred for operation or bathing in 6 cases and for dialysis in one. In 5 cases this strain was isolated from the burn as well as from other sites, in 1 from the upper respiratory tract only, and in 1 from skin only.

Eight members of the staff became infected. The bacteria were, however, only isolated from the upper respiratory tract and there was no clinical infection.

Strains of this phage type were found occasionally on settle plates in the bedrooms, only 1 patient was for a short time dispersing any considerable amount of the bacteria (max. 100 col./plate) and it was found only sporadically on settle plates in the passage. The short episode of dispersal did not coincide with the accumulation of new cases.

DISCUSSION

The main purpose of this investigation was to estimate the risk of cross-infection in an isolation ward for burned patients. During the first year the unit was in use 44 of 69, i.e. 64 % of the burns became infected with *Staph. aureus*. In another 5 patients acquisition of staphylococci could be demonstrated only in specimens from the upper respiratory tract, skin or perineum. As could be expected the infection risk increased when the patients were treated for any length of time in the unit and if the patient stayed more than 5 weeks he was usually infected with 3 or 4 different types of *Staph. aureus*. The infection rate is in agreement with that found by other authors from the same time period (Thomsen, 1970; Wickman, 1970) or maybe slightly less. Of greater interest is to compare our findings with the infection rate found by Körlof in his investigation on burns treated in the plastic surgery unit in Uppsala 1951–5 (Körlof, 1956). The patients were treated in rooms with 1 to 4 beds and no airlocks. He found a *Staph. aureus* infection rate of 83 %. The main principle of treatment was exposure, and the acquisition of *Staph. aureus* occurred within a few days. Thus even if techniques other than isolation have changed since then it seems as though the present ward offers an increased protection against cross-infection. However, it is obvious that cross-infection could not be prevented.

The epidemic situation in the ward was complicated. There were at least three epidemic outbreaks caused by different strains of *Staph. aureus*. In particular one strain, phage type 84, caused a rather extensive epidemic outbreak with no less than 20 infections in burns. Thirty-four members of the staff also became carriers of this strain.

Airborne infection is often considered to be of importance. In this ward high numbers of *Staph. aureus* were often found on settle plates in the bedrooms. In 8 % of the observations there was a dispersal of more than 1800 col./m.^{2} hr. and the maximum value was 14,300 col./m.^{2} hr. Corresponding values in an investigation from an isolation unit for open septic lesions (Williams & Harding, 1969) are more than 283 col./m.^{2} hr. in 8 % of the observations and a maximum value of 2,260 col./m.^{2} hr. It is obvious that patients with infected burns are often heavy dispersers.

A high number of staphylococci in the bedrooms caused an increase in staphylococci in the passage and service rooms. It is, however, difficult to estimate the actual airborne transport of staphylococci from one room to another in this investigation as, especially during an epidemic outbreak, several persons, patients as well as staff, carried *Staph. aureus* of the same phage type and thus possible sources would be found in several rooms in the ward at the same time. In a study of airborne transmission within the ward using tracer particles (Hambraeus & Sanderson, 1972) the transport of particles from one room to another under normal conditions was less than 1 in 4×10^4. According to this investigation it seems likely that other routes of infection have been of greater importance. The significance of this experimentally obtained figure is of course uncertain, and calculation of the airborne transport of staphylococci by studying the ward during selected non-epidemic periods is the object of an investigation now going on.

In the epidemic with type 84 III, the presence of a disperser among the patients did afford a risk, but certainly the risk of transport of bacteria by other routes, such as by nurses' clothes, for example, also increases when the environment is heavily contaminated.

In the 114 bacteriological infections with *Staph. aureus*, 26 of the strains were found in a patient only, 10 in a member of the staff only and 23 in both patients and staff the week before it caused a new infection. Only in a few cases was a patient infected because he shared a room with an infected patient, which seems to indicate the presence of a passive transport of staphylococci from patient to patient.

Carriers among the staff were probably of importance. During two of the epidemics there was a high carrier rate in the staff of the epidemic strain. In the epidemic caused by type 84 a high carrier rate among the staff preceded the epidemic outbreak. During the last part of this epidemic a new increase of carriers among the staff did not result in any new cases among the patients.

It is interesting that many infections occurred after the isolation had been broken for bathing or operation. The bathtub might have been of some importance; *Staph. aureus* of the same type as that causing infections could be isolated from it now and then. The operating ward was regarded as a less important source as

strains common in this ward were hardly ever noticed in other wards served by the same operation unit and team.

It is obvious that with present knowledge no route of infection can be altogether excluded.

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Micropolyspora faeni and farmer's lung disease

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SUMMARY

Several methods were used to obtain serologically active materials from cultures of *Micropolyspora faeni*. From the results of immunodiffusion and immunoelectrophoresis tests on these materials it is suggested that preparations for the laboratory diagnosis of farmer's lung disease (FLD) should contain concentrated culture supernatant (CS) and extracts of mycelium obtained by ultrasonic treatment (MU). Although CS and MU have many serological activities in common they also possess activities unique to each.

Extraction of mycelium with trichloracetic acid, boiling water or methanol yielded a product which gave simple patterns in immunodiffusion tests. The products contained little protein but were rich in carbohydrates, particularly arabinose, galactose and glucosamine. A similar material was obtained from a cell-wall preparation by treatment with lysozyme. Antibodies to the serologically active substances in these materials occurred more frequently in sera of patients with FLD than antibodies to any other *M. faeni* antigen.

Attempts to obtain serologically active materials from spores were unsuccessful. Moreover antibodies to *M. faeni* could not be removed from patients' sera by absorption with partially purified spore preparations. It is suggested that the hypersensitivity in FLD arises from exposure to mycelial antigens.

INTRODUCTION

Micropolyspora faeni is a thermophilic actinomycete found in mouldy hay. It is generally believed to be the causative organism of farmer's lung disease (FLD). Antibodies to this organism have been found in sera from most patients with FLD but also in sera from some apparently healthy people and patients with other respiratory diseases (Pepys *et al.* 1963). Antibodies have been found too in cattle suffering from fog-fever (Jenkins & Pepys, 1965).

Despite these limitations clinical diagnosis of FLD is often supported by the detection of humoral antibody to *M. faeni* using immunodiffusion tests. However no general agreement exists on the best method of detecting antibody or, more

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important, on the best method of extraction of antigens from cultures of the organism.

Early work was done with antigens extracted from defatted mouldy hay by 0·5 % phenol-saline (Pepys *et al.* 1963) or by 10 % trichloracetic acid (Kobayashi, Stahmann, Rankin & Dickie, 1964). In later work cultures of *M. faeni* on solid media were extracted with 10 % trichloracetic acid (LaBerge & Stahmann, 1966) and treated by freezing and thawing (Pepys & Jenkins, 1965). From liquid cultures of *M. faeni* antigens have been obtained by concentration of culture supernatant (Jameson, 1968; Walbaum, Biguet & Tran Van Ky, 1969; Fletcher, Rondle & Murray, 1970) or by freezing and thawing cells. It is perhaps unfortunate that different strains of *M. faeni* were used by different workers so that comparison of results is difficult.

We have attempted to isolate different fractions of *M. faeni* using several extraction procedures and to determine the gross chemical composition and cytological origin of the antigens present in these fractions. We hoped to determine the best method of extraction of antigens and to identify the subcellular fraction which contains the antigens to which antibodies are most frequently detected.

MATERIALS AND METHODS

Growth of organisms

M. faeni, strain 1156, was grown in continuous culture using the conditions described by Fletcher *et al.* 1970. Culture from the fermentation vessel was centrifuged for 10 min. at 3,000 rev./min. The cell mass was washed twice with 0·9 % (w/v) saline and dried from the frozen state. The supernatant was filtered through sintered glass and processed immediately.

Antigens from culture supernatant

Culture supernatant (CS) antigens were concentrated by dialysis against 40 % polyethylene glycol (Carbowax 3000, Union Carbide Ltd.). Clarified supernatant was dialysed for 16 hr. against this solution and then for 36 hr. against 0·02 M phosphate buffer, pH 7·2. The procedure was repeated three times and the resulting concentrate (*approx.* 1/10 of original volume) was stored at 4° C. or dried from the frozen state.

In a few experiments CS antigens were prepared from other strains of *M. faeni*. These were strains 5280, 7760 and 9535 isolated from patients' sputum by Dr Moore, Public Health Laboratory, Exeter.

Attempts were made also to precipitate the antigens in culture supernatant using 2 vol. acetone at 4° C. (CA) and to absorb the antigens onto DEAE-Sephadex (Pharmacia Ltd., Uppsala, Sweden). Absorption was done with ion-exchanger equilibrated with 0·02 M phosphate buffer, pH 7·0, and elution was attempted with 2 M-NaCl. Dried eluted material was labelled CD.

Antigens from mycelium

Disruptive procedures

Suspensions of washed mycelium (250 mg./ml.) in 0·1 M phosphate buffer, pH 7·0 were treated at 4° C. for 2 min. at 17·5 kc/sec. using an MSE-Mullard ultrasonic disintegrator. The supernatant was recovered by centrifugation and the precipitate resuspended in buffer and treated again. The second supernatant was recovered, combined with the first and the pooled material dialysed to equilibrium against 0·02 M phosphate buffer, pH 7·0 and dried from the frozen state. This material was called MU.

Similar suspensions of organisms were treated five times by freezing to −25° C., forcing through an X-press (AB-Biox, Sweden; 1 mm. orifice) and warming to 4° C. Supernatants (MX) were recovered by centrifugation and dried from the frozen state.

Extraction procedures

Suspensions of washed mycelium (100 mg./ml.) in 5 % (w/v) trichloracetic acid (TCA) were stirred at 4° C. for 24 hr. The deposit from centrifugation for 10 min. at 3000 rev./min. was re-extracted as before. After centrifugation the first and second supernatants were pooled, dialysed to equilibrium against phosphate buffer and dried from the frozen state to give the material MT.

A similar suspension was boiled for 4 hr. to give the extract MB and a suspension of mycelium (100 mg./ml.) in methanol was stirred 24 hr. at 4° C. to give the extract MM.

Enzyme treatment

Washed mycelium was suspended to a concentration of 100 mg./ml. in 0·1 M phosphate buffer, pH 7·0 containing 50 μg./ml. lysozyme. The suspension was incubated at 37° C. for 1 hr., the supernatant obtained by centrifugation, clarified by filtration through a Millipore filter (pore size 0·2 μm.) and dried from the frozen state. The material was labelled ML.

Antigens from cell walls

Cell wall preparation

The deposit from the preparation of MU antigens was subjected to further ultrasonic treatment until no intact mycelium could be seen by microscopical examination. The treated material was centrifuged, the deposit washed twice with phosphate buffer, pH 7·0 and twice with distilled water. Gram-positive *M. faeni* spores present in the preparation were almost entirely removed by centrifugation at 500 *g* for 1 min. The final supernatant was dried from the frozen state.

Enzyme treatment

Cell wall preparations were treated with lysozyme as described for mycelium. The soluble material obtained was labelled CWL.

Preparation of spores

Cultures were kept at 55° C. for 2–3 weeks to obtain maximum spore production and lysis of mycelium. Centrifugation at 1000g for 10 min. gave a deposit which was suspended in 0·02 M phosphate buffer, pH 7·0 and subjected to 2 min. ultrasonic treatment. Four cycles of low-speed centrifugation and re-suspension in buffer without ultrasonic treatment gave a preparation which appeared microscopically to be essentially Gram-positive spores. The final preparation was suspended in distilled water and dried from the frozen state.

Attempts to obtain soluble extracts of spores by treatment with 10 % (w/v) TCA or lysozyme were unsuccessful and the preparations were used for serum absorption (*vide infra*).

Antisera used

The two sera used contained antibodies to more *M. faeni* antigens than did 50 others tested. Serum H1 was from a patient with FLD. Serum B1 was from a cow with 'fog-fever'. Both sera were originally investigated by Fletcher *et al.* (1970). They were virtually identical in antibody content.

Immunodiffusion tests and immunoelectrophoresis

Immunodiffusion tests were done as described by Fletcher *et al.* (1970). Immunoelectrophoresis was done essentially as described by Scheidegger (1955).

For test CS, MU and MX were used at 25 mg./ml. Other preparations were used at 15 mg./ml.

Chromatography

Extracts were hydrolysed by N-HCl or 0·5 N-H_2SO_4 for 10 hr. or 5 hr. respectively in sealed ampoules at 100° C. Cell walls were hydrolysed by 70 % H_2SO_4 for 16 hr. at 4° C. and, after dilution to 7 % H_2SO_4, for 4 hr. at 100° C. Hydrolysates were neutralized by an anion-exchange resin (De Acidite FF, carbonate form), clarified by centrifugation, evaporated to dryness *in vacuo* and redissolved in distilled water.

Materials were separated by descending chromatography on Whatman No. 4 paper using as solvent ethyl acetate, pyridine, water (2:1:2) as described by Jermyn & Isherwood (1949). Reducing sugars were detected by alkaline silver nitrate (Trevelyan, Proctor & Harrison, 1950) and amino sugars by the method of Partridge (1948).

RESULTS

Immunodiffusion tests on antigen preparations

The antigen preparations available are summarized in Table 1. Line pattern components (lpc) given in immunodiffusion tests by the preparations are shown in Table 2. Numbers given to the various lpc refer to the 'standard system' of Fletcher *et al.* (1970). Certain lpc are grouped together (3/4/5; 6/7; 8/9/10; 11/12; 13/14/15) because it was frequently not possible to differentiate the individual lpc involved. Some lpc (18–22; 27–29) are omitted because they were not regularly detected in these experiments.

Table 1. *Materials obtained from cultures of* M. faeni

Source	Method	Designation
Culture supernatant	Concentration	CS
	Acetone precipitation	CA
	DEAE absorption/elution	CD
Mycelium	Ultrasonic treatment	MU
	X-press degradation	MX
	TCA extraction	MT
	Boiling water extraction	MB
	Methanol extraction	MM
	Lysozyme treatment	ML
Cell wall	Lysozyme treatment	CWL

Table 2. *Immunodiffusion tests on materials obtained from* M. faeni

Lpc*	CS†	CA	CD	MU	MX	MT	MB	MM	ML	CWL
						Material tested				
1	+	+	+	−	−	−	−	−	−	−
2	+	+	+	−	−	−	−	−	−	−
3/4/5	+	+	+	+	+	±	+	±	±	+
6/7	+	+	+	+	+	−	−	−	−	−
8/9/10	+	+	±	+	+	+	+	+	+	+
11/12	+	+	+	+	±	−	−	−	−	−
13/14/15	+	+	+	+	−	−	−	−	−	−
16	+	+	+	+	+	−	−	−	−	−
17	+	+	−	+	±	−	−	−	−	−
23	+	+	+ +	−	−	−	−	−	−	−
24	+	+	+	−	−	−	−	−	−	−
25	+	+	+	−	−	−	−	−	−	−
26	+	+	+	−	−	−	−	−	−	−
30	−	−	−	+	−	−	−	−	−	−

* Lpc, line pattern components in immunodiffusion tests, the numbers as designated by Fletcher *et al.* (1970).

† The abbreviations are explained in Table 1.

It can be seen that CS gave more lpc (21) than any other preparation. The material CA was similar although some of the lpc were more diffuse and difficult to detect. The material CD was less complex than CS and was not considered further as a possible diagnostic reagent. Examination of CS antigens from other strains of *M. faeni* showed minor quantitative but no qualitative differences; this is shown in Pl. 1, fig. 2. Of the mycelial materials, MU was the most complex, giving 16 lpc. It lacked six of the serologically active materials present in CS but gave lpc 30 which was not given by CS. The material MX was less complex than MU and was not considered further. Differences between CS, MU and MX are shown in Pl. 1, fig. 1.

It was concluded that CS and MU were the most complex preparations and that some lpc given by the one were not given by the other and *vice versa*.

Mycelial preparations MT, MB, MM, ML and the cell wall preparation CWL gave essentially similar results. Each preparation contained only some part of the

Table 3. *Rg values of materials detected in antigen hydrolysates*

Preparation	Rg values				
CA	8	22	65	86	111
MU	4	23	69	87	112
MT	8	—	66	87	113
CWL	9	22	65	86	111
Arabinose	—	—	—	—	111
Galactose	—	—	—	85	—
Glucosamine	—	—	67	—	—

lpc 3/4/5 'complex' and lpc 8/9/10. An immunodiffusion result with ML is shown in Pl. 1, fig. 1 and results with MM, MB and CWL are shown in Pl. 1, fig. 3. The significance of these results is considered in the Discussion.

Absorption of serum with spores

Several attempts were made to obtain soluble, serologically active materials from spore preparations. These were unsuccessful. Attempts were made therefore to absorb antibodies from antisera using purified spore suspensions. In a typical experiment 1 ml. antiserum was absorbed with 50 mg. spores for 4 hr. at room temperature. The result is shown in Pl. 1, fig. 4, where it can be seen that no antibodies were absorbed. Experiments using longer absorption times and different conditions (4° C. and 37° C.) confirmed this finding.

Immunoelectrophoresis

The material CS gave more precipitin arcs with serum H1 than did any other extract. Using the nomenclature of Pepys *et al.* (1963) 3 of the arcs were in the A region and 4 in the C region. The material CA also gave arcs in the A, B and C regions but only 8 precipitin lines could be counted. Preparations MU and MX gave strong but diffuse arcs of precipitation in the A and C regions only. Preparations MT, MB and MM gave one clear arc in the A region and one or two indistinct, diffuse arcs in the C region. Materials ML and CWL gave diffuse arcs in the C region. Materials ML and CWL gave diffuse arcs in all three regions.

Chromatography

The Rg values of reducing materials found in hydrolysates of CA, MU, MT and CWL are given in Table 3. In all preparations arabinose, galactose and glucosamine were detected. Other substances with Rg values of 25 or less were also found. These were not identified.

DISCUSSION

This work shows that concentrated supernatants (CS) from cultures of *M. faeni* are serologically more complex than antigen preparations made in other ways. However, certain extracts of mycelium (MU) contain at least one serologically active substance (that giving lpc 30) that is not present in CS. For this reason it is

suggested that diagnostic antigens for FLD should be made by pooling preparations of CS and MU. Such preparations would contain not only materials giving all the known lpc of *M. faeni* extracts in immunodiffusion tests but also those materials giving precipitin arcs in the A, B and C regions in immunoelectrophoresis.

The close serological similarity between CS and MU is at variance with the finding of Walbaum *et al.* (1969). These authors detected only three antigens common to extracellular material and extracts of mycelium. However their results were obtained using sera produced in rabbits by inoculation of material from the different sources. They state that the antisera did not contain antibodies to materials migrating in the C region in immunoelectrophoresis, and this may account in part for the discrepancy between the results. Additionally we have found that rabbits respond poorly to inoculation with materials containing serologically active substances common to CS and MU.

Results with MT, MB, MM, ML and CWL were similar. Each material gave some of the lpc 3/4/5 and 8/9/10 in immunodiffusion tests and gave precipitin arcs in the A and C regions in immunoelectrophoresis. The materials ML and CWL also gave precipitin arcs in the B region. All the materials contained arabinose, galactose and glucosamine. They contained 2–6 % 'protein' as estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). They appear similar in composition and properties to the materials investigated by LaBerge & Stahmann (1966) and were crude fragments of mycelial cell wall. Materials giving lpc 3/4/5 and 8/9/10 in immunodiffusion tests are of interest because the corresponding antibodies are found in 50 % and 57 % respectively of sera from cases of FLD. This is more frequent than antibody to any other antigen.

The failure to extract serologically active material from spores or to absorb antibodies with spore preparations is of interest. It suggests that spore antigens *per se* might not be responsible for the hypersensitivity reactions associated with FLD. Lacey & Lacey (1964) have calculated that approximately $7 \cdot 5 \times 10^5$ actinomycete spores/min. are deposited in the lungs of men doing light work in a heavily contaminated area such as a hay barn. Despite this, spores have been isolated from lung biopsy material in four cases only (Wenzel, Emanuel, Lawton & Magnin, 1964). It is possible that hypersensitivity arises from mycelial fragments carried to the lung adsorbed on spores or that spores rapidly germinate and the mycelium is eliminated on the lung surface.

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EXPLANATION OF PLATE

Fig. 1. The concentrated culture supernatant, CS, gave a complex pattern when tested against the serum H 1. The mycelial extract MU gave a less complex pattern, but both CS and MU contained more different serologically active materials than the mycelial extract MX. The lysozyme extract of mycelium, ML, gave only two or three lpc against serum H 1.

Fig. 2. This figure shows that ultrasonic extracts of four different strains of *M. faeni* gave essentially the same precipitation pattern when tested against serum H 1.

Fig. 3. The extracts MM, MB and CWL gave less complex patterns than the preparation MX. Materials MB and CWL gave three to four lpc whilst MM gave only two to three lpc.

Fig. 4. Absorption of serum B 1 with a spore suspension failed to remove antibodies to serologically active material present in the concentrated culture supernatants CS 1 and CS 2.
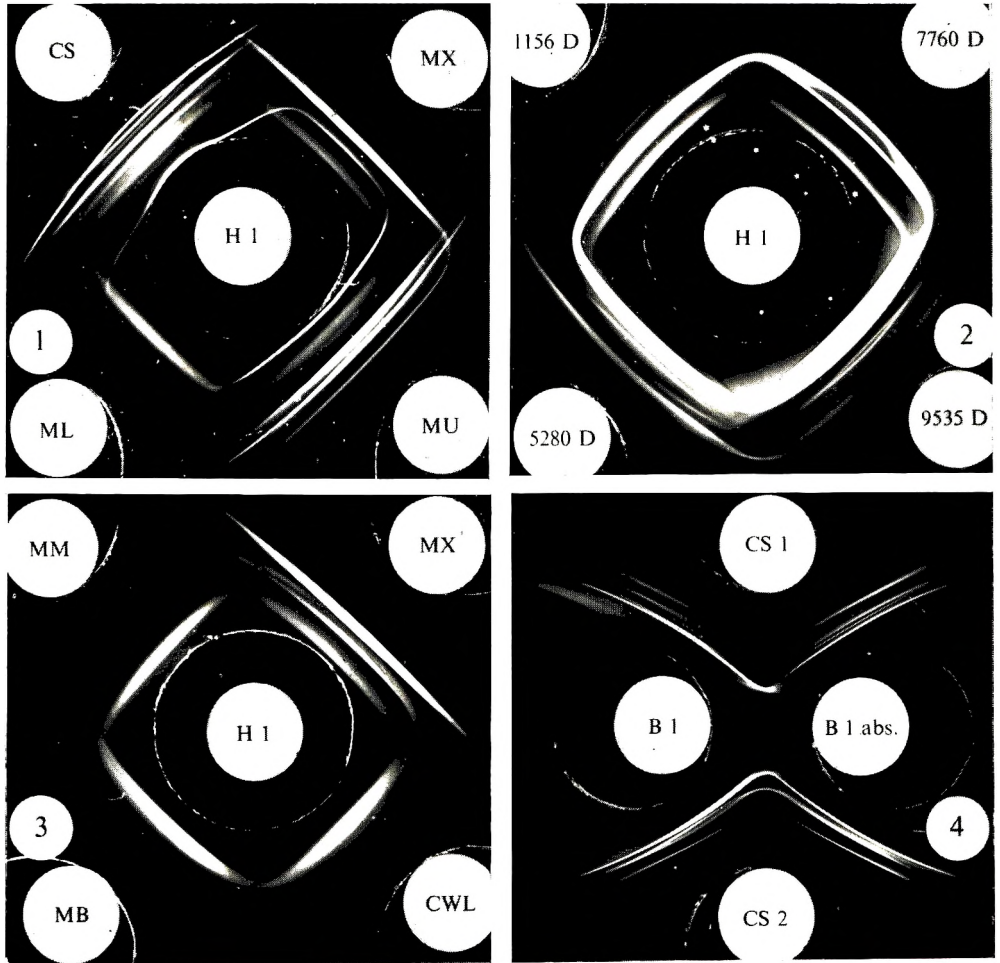
Pertussis antibodies in the sera of children exposed to *Bordetella pertussis* by vaccination or infection

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SUMMARY

Low agglutinin titres to pertussis suspensions were found in 99 % of sera from a group comprising healthy adults and non-vaccinated, non-infected infants of 1–6 months of age. These are attributable to agglutinins to heat-stable antigens and/or heat labile agglutinogen 1, and cross-absorption tests must be done on the sera in order to distinguish between the two. Agglutinins to agglutinogens 2 and 3 were found in only about 20 % of adult sera. Bactericidal antibody was low in titre or absent in all sera from non-exposed individuals.

Raised bactericidal antibody titres and the presence of agglutinins 2 and 3 were attributed to exposure to *Bordetella pertussis* antigens, either as vaccine or as infection. The variation, amongst both vaccinated and infected children, was very great. A vaccinated child who became ill responded to the infection in much the same way as a non-vaccinated child. We were unable to relate the immunity of the child to the titres either of agglutinins or of the bactericidal antibody.

The protective ability of sera from vaccinated or infected children measured in mice against small, lethal brain infections was also unrelated to the state of immunity in the children, but this protective ability was correlated with the complement-mediated bactericidal antibody titres of the sera.

The distribution of agglutinins, bactericidal antibody, and anti-haemagglutinin in serum IgG and IgM was different in vaccinated and infected children.

INTRODUCTION

About 70 sera were sent to us from the Public Health Laboratory Service's investigation in 1967–68 from children ill with whooping cough whose vaccination history was known. Consecutive bleedings from a few of these children were available. The infecting strains had been isolated and typed for agglutinogens, and were reported by the PHLS committee (1969, 1972).

Sera were also collected from vaccinated children who had been exposed to pertussis in a practice in the Guildford area during the 1967 epidemic. Some of these children became ill while others remained well after exposure. Pre-epidemic sera from some of these children were held at the Wellcome Research Laboratories, having been collected as part of a measles vaccination survey 6 months to 3 years previously. Samples from about 60 children and a few adults were investigated.

Sera from vaccinated babies came from a group of 60 in Bristol (1968–70) and from a group of 14 surveyed earlier in Northern Ireland.

13   HYG 71

Base line titres were established on 20 sera from babies and on 100 from healthy adults.

The availability of these sera gave us the opportunity to study pertussis antibody titres in these various categories, particularly in response to exposure to pertussis antigens and their persistence afterwards. Workers in the past have made surveys for purposes of estimating the prevalence of disease in a community, for diagnosis, and for antigen response as a measure both of the effectiveness of a vaccine and, where this is known, of the detection of malfunction of the antibody-forming mechanisms in individual children who do not respond as expected.

Much of the work has, however, been done with very little background knowledge of the kind the results presented here may provide.

A further reason for our interest was to discover if any circulating antibody could be correlated with the state of immunity in the child.

METHODS

Measurement of agglutinins

Members of the genus *Bordetella* contain a number of heat-labile agglutinogens numbered 1–13 (Andersen, 1953; Eldering, Hornbeck & Baker, 1957), of which number 1 is common to all phase I *Bordetella pertussis* and specific to the species and numbers 2–6 are also pertussis-specific, but not necessarily all synthesized by all strains. Agglutinogens 2 and 4 tend to occur together, as do 3 and 6; agglutinogen 5 is relatively rare. The presence in antisera of agglutinins to these agglutinogens 2–6 has been measured by cross-absorption using selected, tested strains which were checked daily for agglutinogen content against our monospecific typing sera prepared by cross-absorption (see below).

Two hundred or so human sera from infected or vaccinated donors were analysed completely and in none was agglutinin 4 found without 2, nor 6 without 3. Out of 51 sera which contained agglutinin 2, agglutinin 4 was also present in only 35 %; out of 92 sera which contained agglutinin 3, agglutinin 6 was also present in only 25 %. Agglutinin 5 was found with 2 in only one child. Since response to agglutinogens 2 and 3 is stronger than to 4, 5 and 6, the measurement and recording of agglutinins to agglutinogens 2 and 3 only are given.

Agglutinations of unabsorbed sera against two suspensions of *B. pertussis* were also done.

The strains used for testing and absorptions were:

(*a*) Strain GL 353, originally from Glaxo Laboratories and containing only very small amounts of specific agglutinogens other than 1, so that in direct agglutinations using unabsorbed sera, titres are largely attributable to reactions with the heat-stable agglutinogens, and to agglutinogen 1.

(*b*) Strain B16, isolated in Northern Ireland, containing agglutinogens 1 and 3, but no demonstrable 6. This was used to absorb agglutinins 1 and 3, or to determine agglutinin 3 in absorbed sera.

(*c*) Strain 3865, first typed in Denmark in 1950, containing agglutinogens 1, 2 and 4. This was used to absorb agglutinins 1, 2 and 4 or to determine agglutinins 2 (plus 4) in absorbed sera.

Agglutinins were titrated in two ways: (i) in 0·3 ml. of serum dilution with 0·3 ml. of suspension of organisms, incubated at 37° C. for 4 hr. in Dreyer tubes, or (ii) in 0·02 ml. of serum dilution with 0·02 ml. of suspension of organisms, incubated at 56° C. for 1 hr. in covered, plastic trays (Disposo trays, Linbro Chemical Co., Inc., 681 Dixwell Avenue, New Haven, Connecticut 06511, U.S.A.). Both tests were held overnight at room temperature before reading.

Method (i) was used in tests for unabsorbed sera; serial doubling serum dilutions were made from 1/5 in saline and incubated with suspension harvested from 20 hr. Bordet–Gengou plates into saline and adjusted to an opacity of 5×10^9 organisms per ml. using the International Opacity Standard. Agglutination end-points were read to the dilution giving a marked agglutination as seen by a hand lens, but just below visibility to the naked eye.

Method (ii) was used to economize on the absorbed sera used for the routine titrations for the determination of agglutinins to heat-labile agglutinogens 2 and 3. Serial doubling dilutions from 1/2 in saline were used in 0·02 ml. volumes measured in '50 dropping pipettes' onto the plastic trays. An equal volume of the suspension, harvested as before, but at 10^{10} organisms per ml., was added. Results were read with a Greenough plate microscope at a twenty-fold magnification. The suspensions used were checked daily for agglutinogens against monospecific pertussis typing sera (prepared by a method to be published by C. J. Shanbury and others). A titre of less than 1/4 was regarded as negative and of 1/4 or more as positive.

Serum absorptions

These were done using (i) suspensions harvested from Bordet–Gengou plates into 0·25 % formol saline, left 24 hr. at room temperature or 2–3 days at 4° C. at kill the cells, and then centrifuged to remove the formol saline. Absorption with B 16 suspension and testing the absorbed serum against 3865 gave the '2,4' agglutinin titre, and absorption with 3865 and testing the absorbed serum against B 16 gave the '3' agglutinin titre; (ii) suspensions similarly harvested, but into saline, the suspensions then being autoclaved at 15 lb. for 30 min. to destroy the heat-labile agglutinogens, and centrifuged.

All suspensions were used wet, $0·5 \times 10^{12}$ organisms to 0·3–0·5 ml. undiluted serum. Sera were absorbed on a turntable rotating at 26 rev./min. (Matburn blood cell suspension mixer, Baird & Tatlock, Chadwell Heath, Romford, Essex) for 3 hr. at 37° C. with each lot of organisms. Three absorptions were used routinely.

Bactericidal antibody

The complement-mediated antibody, killing *B. pertussis in vitro* (Dolby & Vincent, 1965), was tested for by mixing 0·2 ml. of serial fourfold dilutions of antiserum in 1 % Casamino acids with 0·2 ml. of a suspension of living organisms of the antiserum-sensitive *B. pertussis* strain 18–323 and 0·2 ml. of fresh guinea-pig serum (stored at $-15°$ C.) 1/15 as the complement source, making final serum dilutions of 1/30, 1/120, 1/480 and 1/1920. The organisms of 18–323 were harvested from a 20-hr. Bordet–Gengou plate into 1 % Casamino acids and diluted to contain 5×10^6 organisms per ml., about one-tenth of which were viable.

Table 1. *Definition of the bactericidal activity of a serum according to the percentage of organisms killed*

Percentage of organisms killed	Serum dilution		
	1/30	1/120	1/480*
75	−	−	+
85	−	+	
100	+		

* And higher dilution(s).

The mixtures were incubated together in glass tubes on a rotating turntable at 26 rev./min. (see above) for 40 min. at 37° C., and then diluted out for three ten-fold dilutions in 7 % sodium chloride made up in 1 % Casamino acids to stop the bactericidal reaction. Dilutions in 0·02 ml. volumes were pipetted onto Cohen & Wheeler agar plates made with liquid medium (Cohen & Wheeler, 1946) modified by using 0·5 % glutamate and 5 % blood. The colonies were counted after incubating the plates for 4 days at 35° C., and the number of organisms in 0·02 ml. of each final serum dilution was calculated. The maximum count in the 'serum' control and 'complement' control tubes was usually 1500–2500 organisms; the percentage kill at each of the four dilutions of antiserum was estimated. Sera were reported as negative (−) or positive (+) according to the scheme in Table 1, that is for a serum to be considered as positive at 1/30, there must be a 100 % bactericidal effect; positive at 1/120, an 85 % kill, etc. Those positive at 1/1920 were reported as strongly positive (+ +). The titres where given are the highest positive dilution.

Antihaemagglutinin

Serum was diluted serially in two-fold dilutions in 0·02 ml. volumes in plastic trays, as for tray agglutination. Haemagglutinin, contained in whole, freshly harvested (20 hr. Bordet–Gengou plates) cells of *B. pertussis* strain Gl 353, was added in 0·02 ml. volumes. The suspension of Gl 353 was titrated daily in the system and adjusted so as to contain about 2·5 minimal haemagglutinating units against the red cell suspension (see below), usually about 2×10^9 organisms per ml. The mixture of antiserum and suspension was left for 5 min. at room temperature and then a washed, sheep red cell suspension (red cells from 0·1 ml. blood resuspended in 8 ml. buffered saline) added, also in 0·02 ml. The trays were incubated for 2 hr. at 37° C. and read at once by eye, from above, taking complete agglutination as the end-point.

Passive protection tests against a 5000 challenge

Serum dilutions were incubated for 30 min. at 37° C. with the mouse-virulent strain of *B. pertussis*, 18–323. The mixtures, 0·03 ml. of which contained 5000 total organisms of which a tenth were viable (the count was unaffected by incubation with serum), were injected intracerebrally into lightly anaesthetized mice of strain TF1 or Theiler's Original. The organisms were harvested from 20 hr. Bordet–Gengou plates into 1 % Casamino acids.

The effect of the antiserum was determined by estimating the number of live bacteria in the brain at 2 and 5 days after infection and comparing with the number of mice given organisms and normal serum. A 1/10 dilution of antiserum and normal serum was used. A standard rabbit antiserum (6660) at a 1/100 dilution was used as the positive control. Mice were killed with coal gas in groups of five and each brain removed aseptically into 9 ml. of 1 % Casamino acids containing glass beads. The bottles were shaken for 3 min. on a vertical shaker, 325 rev/min., throw $2\frac{3}{4}$ in., and the contents diluted ten-fold and pipetted in 0·02 ml. volumes on to Cohen & Wheeler blood plates (p. 196). Numerical values were given to each antiserum based on the log viable count per brain at 2 days + log viable count per brain at 5 days, abbreviated to 'v.c. 2 + 5'. The value was calculated:

$$\frac{\text{(v.c. } 2+5 \text{ for normal serum)} - \text{(v.c. } 2+5 \text{ for } x)}{\text{(v.c. } 2+5 \text{ for normal serum)} - \text{(v.c. } 2+5 \text{ for standard serum)}}$$

where x is the serum under test. A figure of less than 0·2 was graded as negative (−), 0·2–0·7 as positive (+), and greater than 0·7 as strongly positive (++).

Passive protection tests against a 50,000 challenge

Undiluted sera were mixed with a ten-times stronger challenge than used for the previous test, to give 50,000 total organisms in a mouse dose of 0·03 ml. Organisms of 18–323 were grown and harvested as previously and 0·03 ml. of the incubated serum/challenge mixture (30 min. at 37° C.) injected into Theiler's Original female mice. There was no decrease in viable count due to incubation.

Results were expressed as the percentage of survivors at 14 days and graded as negative (−) for less than 50 % and as positive (+) at greater than 50 %.

Separation of 7S and 19S globulins

This was done as described by Dolby & Dolby (1969). The serum pools were made from individual child sera as follows:

Group A. Eight sera from 5-month-old babies 1 month after two doses of adsorbed triple vaccine. All the sera were positive to both 2 and 3 agglutinogens with titres ranging from 1/16− > 1/640. Antihaemagglutinin was negative in five out of the eight tested; bactericidal antibody was in moderate or high titre.

Group B. Five sera from 7- to 8-year-olds vaccinated 2–8 years previously with unadsorbed triple vaccine. All the sera were positive to agglutinogen 2, but only with low titres of 1/8 and 1/16, and were negative to agglutinogen 3. Antihaemagglutinations were not done on the individual sera; bactericidal titres were low (1/30) on two out of five tested.

Group C. Five sera from 3- to 6-month-old babies infected 2–9 weeks previously. All were positive to agglutinogen 3 with moderate or high titres; two out of five also had agglutinin 2. Antihaemagglutinin was positive in two and negative in two, and bactercidal titres were moderate.

Group D. Five sera from $1\frac{1}{2}$- to 5-year-old children infected 5–14 weeks previously. All had agglutinin 3 and two out of five also had agglutinin 2. Antihaemagglutinin was positive in one and negative in one; bactericidal titres were high.

Table 2. *Pertussis agglutinins in human sera*

	Agglutinins to							
	B. pertussis suspension GL 353				Mixed *B. pertussis* suspension			
Titres	< 1/10	1/10–1/40	1/80–1/160	> 1/160	< 1/10	1/10–1/40	1/80–1/160	> 1/160
Babies 1–6 months	1/20	8/20 40%	9/20 45%	2/20 10%	1/20	13/20 65%	5/20 25%	1/20
Adults 18–60 years	0/50	50/50 100%	0/50	0/50	0/100	6/100	58/100	36/100

Table 3. *Pertussis agglutinins in absorbed human sera*

	Number tested	Numbers of sera with agglutinins to		
		2 only	3 only	Both 2 and 3
Babies 1–6 months	19	0	0	0
Adults 18–60 years	93	14	3	0

RESULTS

Antibody titres in non-vaccinated, non-infected individuals

Agglutinins

Sera from 100 healthy adults between 18 and 60 years of age (history of vaccination or exposure to *B. pertussis* unknown, but only the youngest, born in 1950, were likely to have received vaccine in childhood) and from 20 babies 1–6 months of age were titrated against two *B. pertussis* suspensions: (i) a suspension of *B. pertussis* strain Gl 353 which contains mostly heat-labile agglutinogen 1 with only traces of 2 and 3, and (ii) a mixed suspension of B 16 and 3865 which contains heat-labile agglutinogens 1, 2 and 3; both suspensions contain the heat-stable agglutinogens. Table 2 shows that 100% of adult sera were positive and 19 out of 20 baby sera.

Ninety-three adult sera and the nineteen positive baby sera were then tested for agglutinins to the heat-labile agglutinogens 2 and 3. The sera were absorbed by 1, 2, 0 or by 1, 0, 3 suspensions and then tested for the presence of agglutinin 3 or 2. (As all strains of *B. pertussis* contain agglutinogen 1, agglutinins to 1 are removed from all sera on absorption.) Table 3 shows that only 17/93 adult sera contained agglutinins to 2 or 3 and none of the 19 baby sera were positive. All adult sera, before absorption, agglutinated with *B. pertussis* suspensions; after absorption with organisms containing heat-stable agglutinogens, agglutinogen 1 and either 2 or 3, only 17/93 agglutinated (14 were positive for agglutinin 2 and three for 3), therefore the other 76 sera must have agglutinated with either agglutinogen 1 or the heat-stable agglutinogens.

Agglutinogen 1 and the heat-stable agglutinogens were separated in five adult sera and for comparison seven convalescent child sera all selected as they did not contain agglutinins 2 or 3. The five adult sera were absorbed with autoclaved

Table 4. *Antibody response after pertussis vaccination*

		Babies. Unadsorbed vaccine (see Haire et al. 1967)	Schoolchildren. Various vaccines. Bled between 1965 and March 1967	Babies. Unadsorbed vaccine (see Butler et al. 1969)	Babies. Adsorbed vaccine (see Butler et al. 1969)
Agglutinins*	Sera tested	14	49	14	44
	−	12	31	3	0
	+ for 2	2	15	2	1
	+ for 3	0	2	1	2
	+ for 2, 3	0	1	8	41
Bactericidal antibody*	Sera tested	5	17	11	31
	−	2	5	7	9
	+	1	10	4	11
	+ +	2	2	0	11
Passive protection (5000)*	Sera tested	n.t.	10	3	11
	−	n.t.	4	0	1
	+	n.t.	3	3	5
	+ +	n.t.	3	0	5
Passive protection (50,000)*	Sera tested	n.t.	11	2	4
	−	n.t.	10	1	1
	+	n.t.	1	1	3
Interval between last vaccine dose and bleeding		1–2 months	6 months– 10 years†	1–4 months	1–10 months

* For gradings of response see Methods: agglutinins 2 and 3, page 195; bactericidal antibody, page 196; passive protection (5000), page 197; passive protection (50,000), page 197.
† See text. n.t. = not tested.

organisms, after which they were unable to agglutinate pertussis, therefore the cross agglutination must have been due to heat-stable agglutinogen and its antibody. Of the seven child sera similarly absorbed with autoclaved suspension, three behaved like the adult sera, were not agglutinating and must, therefore, have contained agglutinin only to the heat-stable antigen; four out of the seven were still positive and must have contained agglutinin 1.

To summarize this, human sera fall into three classes: (i) sera with agglutinins to heat-labile 1, 2 and/or 3; (ii) sera with agglutinins to heat-labile 1; and (iii) sera with agglutinins to heat-stable antigens only. In the rest of the paper the response to agglutinogens 2 and 3 only is given.

Bactericidal antibody

None of the nine adults nor the eight babies tested had bactericidal antibodies in their blood according to the definitions of positive and negative set out in Table 1.

Antihaemagglutinin

Twelve out of 12 unvaccinated babies were negative for antihaemagglutinin at a 1/3 dilution.

Table 5. *Antibody titres* of twenty 6-month-old babies bled one month after two injections of one of three vaccines*

	Agglutinins		Bactericidal antibody	Antihaem-agglutinin
	2	3		
Children vaccinated	64	32	480	3
with an unadsorbed	< 4	4	30	< 2
vaccine, dose 20 × 10⁹	20	640	480	n.t.
(group 3, Table 4)	4	< 4	480	n.t.
	16	8	120	n.t.
	40	40	480	> 27
Children vaccinated	4	4	120	< 2
with an adsorbed	128	64	120	< 3
vaccine, dose 20 × 10⁹	512	512	120	> 27
(group 4, Table 4)	> 512	> 512	480	n.t.
	> 512	> 512	> 1920	n.t.
	640	40	120	> 27
	16	320	480	n.t.
Children vaccinated	4	8	120	< 2
with an adsorbed	8	8	1920	3
vaccine, dose 10 × 10⁹	128	32	< 30	n.t.
(group 4, Table 4)	32	128	480	n.t.
	64	32	> 1920	< 3
	40	160	> 1920	< 2
	160	> 640	480	< 3

* Reciprocal.   n.t. = not tested.

The effect of vaccination on pertussis antibody titres in children

Table 4 shows the results of tests on sera from four groups of children given various pertussis vaccines. There were adequate amounts of agglutinogen 2 in all the vaccines, but 3 was low in the vaccines used in group 1, of unknown amount in group 2, and equal to agglutinogen 2 in the vaccines used in groups 3 and 4.

The best response in all the tests was found in babies of group 4 with a maximum interval between the last dose of adsorbed vaccine and bleeding of 10 months. The schoolchildren (group 2) were bled at various times up to 10 years after the last dose of vaccine; a breakdown of these 49 schoolchildren into small groups to show the relationship between the time interval between vaccination and bleeding and agglutinin 2, gave a 30–50 % response from 2 months to 2 years, falling to a 20–30 % response afterwards. Their response of 16/49 in the group as a whole to agglutinogen 2, although less good than in groups 3 and 4, was higher than in the 'normal' population with a figure of 14/93 positive for agglutinin 2 (Table 3). The difference is significant (with a probability of 0·01) and could be attributable to vaccination.

Table 5 shows, however, the enormous variation in the response of individual children of groups 3 and 4 of Table 4 to vaccination with one of three vaccines and also emphasizes again the better antibody response obtained with adsorbed vaccine. Table 5 also demonstrates that high antibody titre to one antigen does not necessarily go hand in hand with high antibody titres to other antigens.

Table 6. *Pertussis antibodies in sera collected at intervals after infection in 3-month to 6-year-old unvaccinated children*

Time from appearance of symptoms to bleed	Serotype of infecting strain	Number of sera	Number of sera possessing		Bactericidal antibody†
			Agglutinin*		
			2	3	
0–1 month	1, 0, 3	16	0	5	8
	1, 2, 3	3	1	0	0
1–2 months	1, 0, 3	13	0	8	6
	1, 2, 3	10	3	4	5
2–6 months	1, 0, 3	3	0	3	2
	1, 2, 3	3	2	3	2

* At titre of 1/4 or more.
† At titre of 1/30 or more (see Table 1).

The consecutive bleedings of a few vaccinated babies were available; individual responses to repeated doses of vaccine varied enormously and in some instances another dose caused a *decrease* of antibody titre. In a group of ten babies bled one month after two doses and one month after three doses of vaccine, six showed increased titres to agglutinins and bactericidal antibody, one showed no change, and three showed decreases.

Antibodies resulting from infection

Table 6 shows the antibody response of 48 Manchester children aged 3 months to 6 years who became infected with strains of *B. pertussis* containing either 1 and 3, or 1, 2 and 3 agglutinogens. Not all the children showed a positive agglutinin response up to a period of 2 months after symptoms. Vaccinated children who became infected behaved similarly to the non-vaccinated ones. Unfortunately, the number of children bled 2–6 months after the appearance of symptoms is too small to show whether the agglutinin response is 100 % by this time, but the proportion having agglutinins at least is certainly increased over those bled before 2 months after onset.

Breaking down the group providing data for Table 6 into age of child reduced the subgroups to very small numbers, but of nine 1- to 3-month-old babies, two out of four had bactericidal antibody within 1 month of infection but not agglutinin 3, whereas by 2 months, three out of four had agglutinin. Three 9-month-old babies were no quicker at antibody production and, in fact, only three out of eight produced agglutinin 3 by 2 months after infection. In 1- to 6-year-old children, three out of five produced agglutinin 3 at 1 month and five out of ten at 2 months.

Without frequent consecutive bleedings, it cannot be determined how much the time of optimum antibody titres following infection varies. Five Manchester children aged 3–6 months were available for this. The results shown in Table 7 indicate wide variation in the time and degree of response to pertussis antigens.

The frequent absence of the agglutinin and bactericidal antibody response after infection was confirmed in a small group of London children with clinical whooping

Table 7. *Pertussis antibodies in sera from consecutive bleeding in non-vaccinated, infected babies*

Sera	Age of child	Serotype of infecting strain	Weeks after infection	Agglutinin*		Bacteri-cidal antibody*
				2	3	
M3/20	3/12	1, 2, 3	4	−	−	−
			8	+	+	+
M24/31	5/12	1, 0, 3	2	−	−	+
			8	−	−	+ +
M32/47	6/12	1, 0, 3	3	−	−	+
			8	−	+	+
M38/44	5/12	1, 2, 3	3	−	−	+
			7	−	−	+
M55/56/89	3/12	1, 0, 3	1	−	−	−
			2	−	+	−
			8	−	+	−

* For gradings of response, see Methods under test for each antibody: agglutinins 2 and 3, page 195; bactericidal antibody, page 196.

cough who were investigated in the first half of 1971. Their ages ranged from 1 month to 5 years, and bleeding times after onset from 2 weeks to $4\frac{1}{2}$ months. Only one, a baby of $5\frac{1}{2}$ months, who had had pertussis for 4 months before being bled, responded with high titres. All the sera had antihaemagglutinin.

Location in the different serum globulins of antibodies elicited by vaccination and infection

Pooled sera from children (five to eight per pool) were separated into 19S and 7S globulins and *in vitro* antibody tests were done to determine the relative efficiency of the fractions from different pools (Table 8).

A group of babies given unadsorbed vaccine produced lower titre sera than a group given adsorbed vaccine but the ratio of activities in the 19S and 7S globulins was similar for both. Results for only the adsorbed vaccine group (A) are given in the table.

A comparison of the 5-month-old vaccinated children bled 1 month after vaccination (group A) with older vaccinated children bled after a longer interval (group B) shows that the antihaemagglutinin titres were similar, but that more bactericidal antibody and agglutinins were in the 7S than the 19S fraction of the baby serum collected soon after vaccination, whilst in older children with a longer vaccination-bleed interval, the 19S was the more active for bactericidal antibodies. Time intervals between vaccination and bleed were more likely to be responsible for these differences than age of child.

The infected babies had higher antihaemagglutinin titres in the 19S than the 7S globulins (group C), while in the older children (group D) infection produced similar antihaemagglutinin levels in both globulins. Levels for other antibodies following infection are similar in both groups in the 7S and 19S fractions.

The type of vaccine, plain or adsorbed, therefore seems to influence only the

Table 8. *Pertussis antibodies in the* 19S *and* 7S *globulin fractions of pooled child sera collected after vaccination or infection*

Donors of serum	Interval between exposure and bleeding and number of samples in pool	Globulin fraction	Total serum protein (%)	Minimal effective concentrations of serum protein			
				Agglutinins (mg./ml.)		Bactericidal antibody (μg./ml.)	Antihaemagglutinin (μg./ml.)
				2	3		
Group A 5-month-olds, vaccinated with 2 doses of adsorbed triple vaccine	1 month (8)	19S	17·2	> 5·0	1·25	68	200
		7S	11·8	1·25	0·3	14	> 1700
Group B 7 to 8-year-olds, vaccinated with unadsorbed triple vaccine	2–8 years (5)	19S	18·8	> 5·0 } n.t.		{ 340	300
		7S	24·0	> 5·0 }		{ 1700	> 1700
Group C 3 to 6-month-olds, infected with 1, 3 strains	2–9 weeks (5)	19S	15·6 } n.t.		{ 1·25	340	170
		7S	12·9 }		{ 2·5	340	850
Group D 1½ to 5-year-olds, infected with 1, 3 strains	5–14 weeks (5)	19S	18·0 } n.t.		{ 1·25	13·4	42
		7S	18·7 }		{ 2·5	13·4	42

n.t. = not tested.

Table 9. *Pertussis antibody titres* of six children from whom blood samples were available before and after known exposure to a* 1, 0, 3 *strain of* Bordetella pertussis

Serum number	Time from vaccination to 1st bleeding	1st bleeding titres*					2nd bleeding titres*			
		Agglutinin		Bactericidal antibody	Time from 1st bleed to exposure	Clinical pertussis	Time from exposure to 2nd bleed	Agglutinin		Bactericidal antibody
		2	3					2	3	
WP 138/G 134	10 months	< 4	< 4	> 1920	1 year	+	6 weeks	< 4	< 4	480
WP 50/G 39	7½ years	< 4	< 4	30	5 months	+	4 weeks	< 4	< 4	480
G 128/G 58	7 months	256	< 4	120	3 years	−	1 week	> 8	4	120
WP 67a/b	1½ years	16	< 4	480	2 months	−	2 years	16	< 4	120
WP 101/G 109	3 years	< 4	< 4	> 1920	2 years	−	3 months	< 4	< 4	480
WP 28/G133	5½ years	< 4	< 4	120	6 months	−	6 months	512	< 4	n.t.

* Reciprocal.

antibody titres, not the ratio in 19S and 7S globulins; the time interval between vaccination and bleeding, and the age of the child, and whether exposure to pertussis antigens is in the form of vaccine or infection, do influence the ratio.

Antibodies in sera of children exposed to infection

Table 9 gives the details of six children in the Guildford series for whom pre-exposure and post-exposure serum samples were available; unfortunately, these were the only children from whom both pre- and post-samples were obtained. Table 10 gives details of 27 children and six adults from whom only one serum

Table 10. *Pertussis antibody titres* of 33 children and adults from whom blood samples were available either before or after known exposure to a 1, 0, 3 strain of* Bordetella pertussis

	Serum number	Time between — Vaccination and bleed	Bleed and exposure	Exposure and bleed	Clinical pertussis	Agglutinin 2	3	Bacteri-cidal antibody
Pre-exposure samples	WP 140	6 months	1 year	.	+	32	< 4	30
	WP 167	1½ years	5 months	.	+	< 4	< 4	n.t.
	WP 27	4½ years	6 months	.	+	4	< 4	120
	WP 127	2 years	1 year	.	+	64	< 4	120
	WP 175	3 years	3 months	.	+	4	< 4	480
	WP 128	1 year	1 year	.	+	64	4	120
	WP 65	4 years	4 months	.	−	< 4	< 4	30
	WP 29	5½ years	6 months	.	−	< 4	< 4	480
	WP 30	5½ years	6 months	.	−	< 4	< 4	480
	WP 148	2½ years	1 year	.	−	< 4	< 4	120
Post-exposure samples	G 92	3 years	.	2 days	+	< 4	< 4	120
	G 91	2 years	.	2 days	+	< 4	< 4	n.t.
	G 51†	.	.	1 week	+	8	< 4	n.t.
	G 93†	.	.	3 days	+	8	< 4	30
	G 101	10 years	.	1 week	+	< 4	< 4	120
	G 95	2 years	.	1 week	+	128	< 4	480
	G 98	2 months	.	2 weeks	+	< 4	16	> 1920
	G 102	3 weeks	.	2 weeks	+	> 512	< 4	> 1920
	G 108	1 year	.	1 month	+	128	< 4	480
	G 106	5 weeks	.	1 month	+	< 4	< 4	480
	G 107†	.	.	3 months	+	8	< 4	120
	G 116	10 years	.	3 months	+	< 4	< 4	> 1920
	G 113	3 years	.	3 months	+	< 4	< 4	480
	G 114	2 years	.	3 months	+	< 4	< 4	n.t.
	G 126	3½ years	.	3 months	+	< 4	< 4	480
	G 127	1 year	.	3 months	+	16	< 4	480
	G 96	5 years	.	2 weeks	−	4	< 4	120
	G 105†	.	.	2 weeks	−	32	4	480
	G 94	3 years	.	4 weeks	−	< 4	< 4	480
	G 130†	.	.	4 weeks	−	< 4	< 4	120
	G 110	3 years	.	3 months	−	< 4	< 4	480
	G 132	1½ years	.	3 months	−	64	< 4	120
	G 129†	.	.	3 months	−	< 4	< 4	480

* Reciprocal.   † Adults.   n.t. = not tested.

sample was available, ten children (of whom six developed clinical pertussis) with pre-exposure samples and the rest (16 developed clinical pertussis, seven though exposed did not) with post-exposure samples only. These were children in a suburban practice and there was great variation in age and in details of time between vaccination and exposure and in the relative times the bleedings were taken.

It will be seen from these tables that there is no consistent difference between pre-exposure titres and post-exposure titres, nor in the titres of infected or non-infected children. It is impossible to tell from the results the difference between those resisting infection and those not, either before or after exposure. Passive protection tests against a 5000 challenge done in the ten 'pre-exposure only' sera could not distinguish between them.

The passive protection tests and correlation with bactericidal complement-mediated antibody, acting in vitro, *and the immune state of the child*

Some of the results for sera tested for ability to protect against a 5000 challenge when introduced into the brain with the infection, as measured by a reduction in bacterial numbers, are shown in Table 4. The antibody is usually present soon after vaccination (groups 3 and 4) and is still present in some vaccinated children (group 2). On the small number of tests done on sera described in the last section, ability to protect against this small challenge does not distinguish, however, between protected and non-protected children.

To determine whether the bactericidal *in vitro* antibody (which also did not seem to be correlated with child protection) was the one responsible for the reduction in numbers in the mouse brain, the results on the 41 sera tested for their ability to reduce a 5000 challenge in the mouse brain were compared with their *in vitro* performance in the presence of complement; when each individual passive protection index (see Methods for formula) was compared with the $\log_{10}$ of the inverse of each bactericidal titre, a correlation coefficient of 0·57 was obtained. If constants are used for both the normal and standard sera, calculated by averaging these two values for all the tests done, then the correlation coefficient is increased slightly to 0·67; the correlation is only moderate.

Passive protection tests were done on a small number of sera with a challenge of 50,000 organisms, mixing challenge and serum and measuring protection as the percentage of mice surviving 14 days. This antibody was present in 4/6 children soon after vaccination (Table 4). It was absent in the sera of 17 infected children, each bled once, at 1–12 weeks after infection, but present in two out of three children who had been exposed but remained well (sera G 109 and G 96 positive, G 94 negative, Tables 9 and 10). In the few sera tested, this antibody was unrelated to any other measured.

DISCUSSION

The finding that 99 % of unabsorbed human sera had agglutinins to pertussis and that low bactericidal activity was common made us wonder if there may not be a heterophile antigen involved. The sera used included adult sera and sera from babies. Without absorption, it was impossible to tell whether the positive agglutination was due to the presence of agglutinins to heat-stable antigen(s) of pertussis or to the heat-labile agglutinogen 1 common to all *B. pertussis* (Andersen, 1953). Our results with a few sera suggest that agglutinin 1 is present only after fairly recent contact with pertussis, and that the widely distributed agglutinin was to heat-stable antigen(s).

These agglutination titres of 1/40–1/320 with even normal sera are stronger than those reported by Kendrick *et al.* (1969) and different from the uniform negatives reported by others (Abbott, Preston & MacKay, 1971; Raška, 1971). Our tests were done in several ways using both tubes and trays, and the low positive titres were independent of technique. These agglutinations, however, were not of the strong type associated with agglutinogens 2 and 3, so the difference may be one of degree of reaction, a point emphasized in a general discussion on agglutination

end-points by G. Eldering (personal communication). The other consideration is that only four strains of pertussis were used in these tests: Gl 353, 18–323, B 16 and 3865; recently, Dr Holt (personal communication) has found that some strains of pertussis are easily agglutinated by 'normal' serum and others are not, so we may have selected strains with a lot of heterophile, heat-stable antigen. The finding of Muschel & Osawa (1959) that *Escherichia coli* 'bactericidal antigen' and human group B red cells cross-react, and that of Ackers & Dolby (1972) that pertussis 'bactericidal antigen' and sheep red cells cross-react, may not be irrelevant.

The above observations, and those of the great variations in the response of children to both vaccination and infection, should be taken into account in attempting to assess the value of vaccination (Butler *et al.* 1969; Abbott *et al.* 1971) and in the diagnosis of disease (Cruickshank *et al.* 1970) by the assay of antibodies.

Attention had previously been drawn to variation in response to vaccine by Dane *et al.* (1966). Dr C. B. Wood (personal communication) has pointed out that the antibody titres of the sera of vaccinated babies, of which the data given in Table 5 are an example, do not fall on a Poisson distribution curve, but into groups of good and bad responders.

There is a tendency, pointed out by the Scottish group (Cruickshank *et al.* 1970), which we have also observed, that older children do, on the whole, respond better than young ones, but this cannot be applied to individuals; a 4-week-old baby may respond better, for example, to the agglutinogen of his infecting strain than a 12-year-old.

Whether our failure to detect an antibody on which the immune state depends is a basic one, simply because this is not the mechanism of child immunity to pertussis, only future work can show. That the failure was because we were measuring the wrong antibodies, or measuring them at the wrong time, is a possibility. It is hoped that the passive protection test against a large infection can be done on more samples available in the future.

The response to infection may be quite different from that to vaccination – the distribution of antibody activities amongst the serum globulins suggests this. The extension of such tests and the inclusion of 11S antibody may be worth while. Bactericidal antibody, for instance, is present in much higher amounts in the 7S than the 19S fractions in rabbits, mice and children injected parenterally, but children infected have high equal amounts of 7S and 19S, and antihaemagglutinin is present in both globulins.

Part of this work has been submitted by one of us, S. S., as a Thesis for membership of the Institute of Biology.

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Transmission of infectious drug resistance from animals to man

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SUMMARY

The antibiotic resistance patterns of coliforms in faecal specimens from pigs and their human contacts were studied. The ability of the resistant coliforms to transfer their resistance *in vitro* to antibiotic-sensitive recipients was examined. The results showed that pigs which had received antibiotics carried more multiply-resistant, R-factor bearing coliforms than pigs which had not been given antibiotics. Human contacts of the antibiotic-treated pigs had a higher incidence of antibiotic-resistant coliforms with R-factors than human contacts of pigs which had not been given antibiotics. It is concluded that antibiotic treatment of farm animals may lead to acquisition of antibiotic resistance by gut coliforms of man.

INTRODUCTION

Infectious drug resistance was first noted by the Japanese (Ochiai, Yamanaka, Kimura & Sawada, 1959) who observed the transfer of antibiotic resistance from *Escherichia* to *Shigella* in patients with dysentery. Infectious drug resistance is of widespread occurrence (Datta, 1962; Maré & Coetzee, 1965; Mann & Gebedou, 1966; Schroeder, Terry & Bennett, 1968; Anderson & Lewis, 1965a; Anderson, 1968) and is mediated by R-factors which are extra-chromosomal elements consisting of deoxyribonucleic acid (Falkow, Citarella, Wohlhieter & Watanabe, 1966). R-factors may be transferred from antibiotic-resistant bacteria to antibiotic-sensitive bacteria during conjugation (Ochiai *et al.* 1959); the recipients of R-factors become potential donors so that these factors may spread rapidly. The origin of R-factors is not known but their selection and spread appear to be promoted by the widespread use of antibiotics in human and veterinary medicine (Mann & Gebedou, 1966; Smith, 1966). Walton (1966) found a high incidence of infectious drug resistance in strains of *E. coli* isolated from the faeces of healthy pigs and calves and was able to correlate this with antibiotics given in the feeds.

The indiscriminate use of antibiotics as feed additives and for prophylaxis in animal farming has come under scrutiny and criticism by Anderson & Lewis (1965a), who suggested that normally harmless bacteria of animal origin could develop drug resistance as a result of exposure to antibiotics and transfer this resistance to normal gut commensals in humans. The mechanical transmission of organisms from animals to man could result from direct contact with the animals

or from eating animal products, e.g. sausages (Moorhouse, O'Grady & O'Connor, 1969). These resistant gut commensals could then act as reservoirs capable of transferring their drug resistance to bacterial pathogens during subsequent infections.

This investigation was an attempt to study the transmission of drug-resistant bacteria from pigs to man by ascertaining:

(1) the incidence of antibiotic-resistant bacteria in the gut coliforms of pigs and their human contacts;

(2) any similarity in the antibiotic resistance patterns of the coliforms from pigs and their human contacts;

(3) whether these antibiotic-resistant bacteria could transfer their resistances *in vitro*.

MATERIALS AND METHODS

Specimens

Rectal swabs were taken from 110 pigs known to have received antibiotics as injections or with their feeds. The antibiotics were penicillin, chlor- and oxy-tetracyclines, dihydrostreptomycin and sulphamethazine. Faecal samples were also taken from 42 human contacts of the pigs (handlers and their close relatives).

A control group consisted of faecal samples from 72 pigs, not known to have received any antibiotics, and from 28 human contacts of these pigs. All of these contacts had at some time handled the pigs so that there is no distinction between handlers and their relatives in this group.

Processing of specimens

These were plated on MacConkey agar and their sensitivity to the following antibiotics determined by the Bauer–Kirby technique (Bauer, Kirby, Sherris & Turck, 1966); ampicillin (A) 25 μg., streptomycin (S) 25 μg., tetracycline (T) 50 μg., chloramphenicol (C) 50 μg., sulphatriad (Su) 300 μg. and nalidixic acid (NA) 30 μg. per disk. Resistant colonies were identified by conventional procedures and picked to Mueller–Hinton broth to be used as donors in mating experiments. All donor strains were sensitive to nalidixic acid while the recipient was a multiply-sensitive strain of *E. coli*, resistant to nalidixic acid only.

Equal volumes of 0·5 ml. of 18 hr. broth cultures of donor and recipient were mixed in tubes and 4·0 ml. tryptose broth added. The mixture was then incubated for 3 hr. at 37° C., after which approximately 0·2 ml. was plated on MacConkey agar plates containing nalidixic acid (25 μg./ml.) plus streptomycin (10 μg./ml.) or tetracycline (10 μg./ml.). On these plates neither the donor nor the recipient could grow, so that organisms recovered from the mating mixture on these plates were considered recombinants. These were picked to fresh Mueller–Hinton broth and their antibiotic sensitivity determined. All antibiotic resistances acquired by the recipient strain were considered to have been transferred from the antibiotic-resistant donor.

Table 1. *Multiple drug resistance of coliforms isolated from*
pigs and their human contacts

No. of antibiotics to which coliforms resistant	CP 72	TP 110	CH 28	TH 42	EM 14	RE 28
3 or more	18 (25)	106 (96)	0	24 (57)	12 (86)	12 (43)
2	20 (28)	4 (4)	10 (36)	8 (18)	2 (14)	6 (21)
1	30 (41)	0	10 (36)	4 (9)	0	4 (14)
0	4 (6)	0	8 (28)	6 (15)	0	6 (22)

Figures at top of table indicate total number of individuals in each group; figures in parentheses are approximate percentages.
See text for explanation of abbreviations.
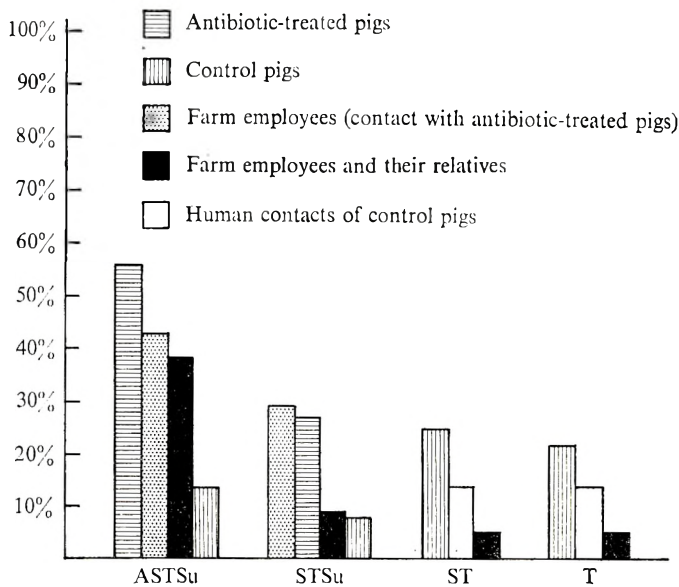
Fig. 1. Patterns of antibiotic resistance.

RESULTS

Resistance patterns of the strains

Of the specimens from the antibiotic-treated pigs (TP), 96 % carried coliforms resistant to more than three antibiotics as against only 25 % from the control pigs (CP).

Of the human contacts of the antibiotic-treated pigs (TH), 57 % carried coliforms resistant to more than three antibiotics as against none of the contacts of the control pigs (CH). Of the farm employees who had direct contact with the antibiotic-treated pigs (EM) 86 % carried coliforms resistant to more than three antibiotics while of their relatives, who had only indirect contact with the pigs (RE), 43 % carried coliforms resistant to more than three antibiotics. These results are summarized in Table 1.

Table 2. *Antibiotic resistance to individual drugs in test and control groups*

	CP	TP	CH	TH
Resistant to:	72	110	28	42
Ampicillin	10 (14)	78 (71)	2 (7)	20 (48)
Streptomycin	36 (50)	104 (95)	8 (29)	30 (72)
Tetracycline	52 (72)	100 (91)	12 (43)	26 (62)
Sulphonamide	36 (50)	102 (91)	8 (29)	32 (76)
Chloramphenicol	2 (3)	0	0	0
Sensitive to all	4 (6)	0	8 (29)	6 (14)

Figures at top of table indicate total number of individuals in each group; figures in parentheses are approximate percentages.

See text for explanation of abbreviations.
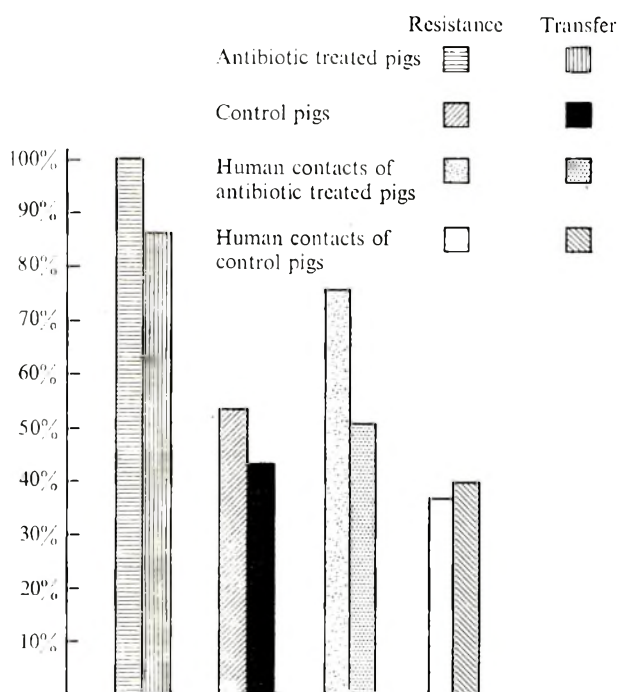
Fig. 2. Antibiotic resistances and transfer of two or more drugs. The left-hand column in each pair shows the percentage of resistant strains, the right-hand column the percentage of these resistant strains which had transferable drug resistance.

The commonest combinations of antibiotics to which resistance was noted were ASTSu, STSu, ST and T only. Antibiotic-treated pigs and their human contacts had a higher incidence of the ASTSu combination than the control pigs and their human contacts. These results are shown in Fig. 1.

The commonest resistance pattern was STSu, and the incidence of this pattern was higher in the antibiotic-treated group than in the controls. These results are summarized in Table 2.

Transferability of resistances

Of the resistant strains from the antibiotic-treated pigs, 86 % transferred resistance to two or more antibiotics. By contrast, only 42 % of the resistant isolates from the control pigs transferred resistance to two or more antibiotics. Of the resistant isolates from contacts of the antibiotic-treated pigs, 50 % transferred resistance to two or more antibiotics as against 40 % of the resistant isolates from contacts of the control pigs. The results are represented in Fig. 2.

DISCUSSION

All the antibiotic-treated pigs carried coliforms with resistance to two or more antibiotics as against 53 % in the control pigs. This confirms the effect of antibiotic exposure on the selection of antibiotic-resistant bacteria. Resistance was commonest to those antibiotics to which the animals had been exposed. It was interesting to note that although none of the antibiotic-treated pigs had received ampicillin (they received penicillin) yet resistance to ampicillin was frequent in the antibiotic-treated pigs and their human contacts. A possible explanation of the relatively high incidence of antibiotic resistance in the gut flora of the control pigs (53 % resistant to two or more drugs) is that some of the pigs may have received antibiotics unknown to their owner, e.g. before purchase. It is also possible that animals may acquire R-factors by ingestion of vegetation containing antibiotic-producing organisms.

All the handlers of the antibiotic-treated pigs carried coliforms resistant to two or more antibiotics; 64 % of the relatives of these pig-handlers also carried coliforms resistant to two or more antibiotics as against 36 % of the human contacts of the control pigs. Thus, a lesser degree of contact with antibiotic-treated pigs was paralleled by a lower incidence of gut coliforms with multiple drug resistance. The similarity of the patterns of antibiotic resistances (ASTSu, STSu) between the antibiotic-treated pigs and their contacts is impressive and these results suggest that multiply-drug resistant organisms are transmitted from the animals to man. All of the handlers of the antibiotic-treated pigs were healthy young men, none of whom had received antibiotics within the previous 2 years and most of whom denied ever having received any antibiotics to their knowledge.

Strains from 78 % of the control pigs and 79 % of their human contacts did not exhibit infectious drug resistance. This contrasted with 14 % of the strains from the antibiotic-treated pigs and 53 % from their human contacts. Although the resistance patterns were similar in the antibiotic-treated pigs and their human contacts, yet the coliforms from the latter transferred their resistances at a much lower rate than those of the pigs (47 % against 86 %). This may point to some relative instability in the human gut of the Resistance Transfer Factor (RTF) from animal gut flora, a possibility supported by the findings of Smith (1969a, b). Without the RTF, an antibiotic resistance may be expressed but not transferred; for transfer of drug resistance both the resistance determinants and a transfer factor must be present (Anderson & Lewis, 1965b).

Another interesting observation made during this study was that as the pigs got

ANTIBIOTIC

↓

Animal gut flora

↓

Antibiotic-resistant coliforms with
R-factors in animal guts

|

Ingestion of animal
products/human contact

↓

Antibiotic-resistant coliforms with
R-factors in human gut

|

Transfer of R-factors by conjugation

↓

Antibiotic-resistant human coliforms
(reservoir of R-factors)

|

Conjugation with antibiotic sensitive
Salmonella/Shigella

↓

Antibiotic-resistant human pathogens

Fig. 3. Possible mode of transfer of multiple drug resistance from animals to man.

older and were moved to a different section of the farm (from 'nursery' – where they all received antibiotics, to 'finishing' to 'gestation' to 'farrowing' – where they infrequently received antibiotics) the incidence of multiple drug resistance diminished slightly, while the ability to transfer the resistances diminished much more. This was some indication of reduced antibiotic exposure being paralleled by reduced incidence of multiple infectious drug resistance.

Our findings suggest that widespread use of antibiotics on animal farms can lead to the development of highly drug-resistant bacteria in the gut flora of the animals. These bacteria may be transmitted to human contacts who will then harbour resistant coliforms in the gut. Some of these may retain the transfer factor and be capable of passing on their resistance to initially sensitive bacterial pathogens with which the subject may become infected. The possible course of events is shown in Fig. 3.

It is recommended that antibiotics be used only when absolutely necessary, for therapeutic rather than prophylactic purposes. When the circumstances dictate, they should be given in adequate dosage for an adequate length of time. By these means one can minimize the emergence of antibiotic-resistant organisms which so often destroy the efficacy of antibiotics in the treatment of infection.

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