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The epidemiology of *Salmonella dublin* infection in a dairy herd

I. Excretion and persistence of the organism

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(*Received* 17 *August* 1973)

SUMMARY

This paper describes the epidemiologically relevant events that took place in a dairy herd infected by *Salmonella dublin*. The evidence presented indicates that it may be possible to eliminate infection from the farm and that residual infection or persistent excretion are uncommon. In two animals infection persisted, in one instance in the tonsil and in the other in the gall bladder. In this latter case the infection remained from the neonatal period until adulthood. It is possible that both these animals are relevant in a more general context and are indicative of the source of infection in outbreaks in which the origin of infection cannot be determined by more routine examinations.

INTRODUCTION

Clinical salmonellosis of cattle is most commonly caused by *Salmonella dublin* or *Salmonella typhimurium*. Whilst the sources of the latter organism are many and varied *S. dublin* is more restricted in its distribution and may be considered as relatively host specific (Report, 1965).

During the investigation of outbreaks of *S. dublin* infection many authors have been unable to detect a possible source of infection. Thus Meissner & Koebe (1931), Craig, Davies & Massey (1941), Field (1948) and Gibson (1961) failed to detect carrier animals in outbreaks of calf salmonellosis, while Bythell (1946), Field (1948), Grunsell & Osborne (1948), Barron & Scott (1949) and Smith & Rutherford (1965) were sometimes unsuccessful in their attempts to determine the likely source of infection in salmonellosis of adult cattle. These observations indicate deficiencies in our knowledge of the epidemiology of *S. dublin* infections in cattle.

Since December 1969 we have had the opportunity to study in some detail a closed herd of dairy cattle, in which *S. dublin* infection was present. The following account is a description of the steps taken in attempting to eliminate the infection from the herd and the relevant epidemiological observations made during this period. The serological tests carried out during this investigation are the subject of a separate paper (Lawson, Wooding & McPherson, 1974).

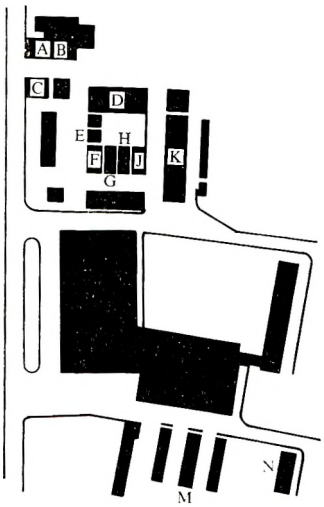

Fig. 1. Plan of infected farm and adjoining premises. *A*, emergency calf house; *B*, isolation byre for cows; *C* and *E*, calf houses; *D*, byre; *F*, *G*, *H* and *J*, court accommodation; *K*, piggery; *M* and *N*, detached calf accommodation.

The farm and stock

The farm steading is a mixture of traditional farm buildings and a newer but largely standard byre, court and calf house.

The principal livestock maintained on the farm are a dairy herd of Ayrshire cattle, flocks of Cheviot and Suffolk sheep, a hysterectomy-derived pig unit and a flock of laying hens. The Ayrshire herd consists of between forty and fifty milch cows and their replacement stock. Male animals have been introduced infrequently, the last introduction being an adult bull in January 1968. In addition to the largely self-contained dairy herd, a number of home-bred Ayrshire and cross Shorthorn calves and occasional batches of purchased calves, have been fattened for beef. The purchased calves which originated from various sources were assembled and then maintained at a rearer's premises until at 6 weeks of age they were moved to the farm.

The farm is similar in character to other dairy farms in the Southern Uplands of Scotland, but, for various reasons, the movement of stock and passage of personnel within the farm is greater than in most herds. There is additional animal accommodation (*M* and *N*) housing calves occasionally, close to the farm but separated from it by buildings not used to accommodate livestock (Fig. 1). The farm and the premises *M* and *N* are largely self-contained areas, but there is always some traffic, mainly of persons and vehicles, between the two.

The farm had a previous history of salmonellosis dating back to 1963. In October/November of that year 62 six-week-old cross Friesian calves were brought into the farm for beef production. Shortly after their arrival mortality occurred associated with *S. dublin* and following rectal sampling three calves were identified as being infected with *S. dublin* and one calf with *S. typhi-murium*: this latter organism has not been encountered on the farm since that isolation. Excretion in this group of calves appeared to have ceased by January 1964 and three clear

samples were obtained from all these calves between January and March 1964. In January and February of the same year, infection with *S. dublin* had spread into the home-bred calves, two of which died and four others excreted the organism. These latter calves were removed to other premises and eventually slaughtered in June of that year. Limited sampling after this time appeared to indicate that excretion had ceased and at no time from 1964 until the outbreak in 1969 was *S. dublin* encountered either in the routine necropsy of calf deaths or in bacteriological samples taken for diagnostic or other purposes from the farm. During the period in question all cattle, sheep or pigs dying on the farm were subjected to post-mortem examination and ancillary bacteriological examination.

Endemic fascioliasis, which may influence the carrier state in salmonellosis (Frik, 1969), is not present on the farm.

MATERIALS AND METHODS

Bovine faecal samples

Calves less then 4 months old were sampled by rectal swabbing using cotton wool swabs. Other young stock and adult cattle were sampled from the rectum using individual polythene gloves. Faecal swabs were placed in 10 ml. volumes of Selenite F broth (Cruickshank, 1965). Faecal samples from older cattle were handled in a variety of ways: (1) an estimated 2 g. sample was inoculated into 10 ml. of Selenite F broth or (2) an estimated 20 g. sample was placed in 100 ml. of Selenite F broth, or (3) two to five samples of approximately 5 g. each from different animals were introduced into 100 ml. of Selenite F broth. In many cases samples were examined by methods (1) and (2) simultaneously. Selenite broths were inoculated as soon as possible after taking the sample, and generally within 3 hr. of sampling. Method (3) was employed for herd tests and where positive results were obtained further individual samples were taken and examined. 100 ml. volumes of Selenite F broth were dispensed in 300 ml. wide-mouth jars with 'twist off' caps.*

Drain swabs

Gauze drain swabs (Moore, 1948) were used and left in position for 24 or 48 hr. before being transferred to 100 ml. volumes of Selenite F broth or Selenite F broth containing 1 % lauryl sulphate (Jameson, 1961).

Placentae

A large portion of placental tissue was introduced into 100 ml. Selenite F broth.

Milk samples

Aseptically taken quarter samples from groups of 5 cows were bulked and 100 ml. of milk inoculated into 100 ml. of double strength Selenite F broth.

Bovine tissues

All cattle that have left the farm since infection was first detected have been examined bacteriologically at slaughter. Healthy animals were transported to the Edinburgh Corporation Slaughterhouse direct from the farm and slaughtered

* 'Vapour Vacuum', 66 Whitecap International Ltd., Poole, England.

within 2 hr. of their arrival (with the exception of two animals) at the abattoir. The apparently normal animals were killed and dressed either in booths or in the line system; the others were killed in the slaughterhouse isolation block for a variety of reasons including potential infection.

The following tissues were removed as soon after slaughter as possible and conveyed to the laboratory in individual polythene bags; both tonsils, all the hepatic lymph glands, all the mesenteric lymph glands, the superficial nodes of the colic lymph glands (Sisson, 1963), the last 46 cm. (approx.) of ileum and the gall bladder. The portion of intestine and the gall bladder were ligated before removal from the remainder of the viscera.

At the laboratory the lymph nodes were dissected free of fat, flamed twice to extinction after immersion in methyl alcohol for periods of 30 sec., ground with sand in a mortar and inoculated into an appropriate volume of Selenite F broth. Hepatic and colic lymph nodes were inoculated into one or more 10 ml. amounts of broth and tonsils and mesenteric lymph nodes were inoculated into one or more 100 ml. volumes of Selenite F; approximately 100 ml. of bile were added to 100 ml. of double strength Selenite F broth. The portion of small intestine was opened longitudinally, washed gently in running water and the mucosal surface dried with a paper towel. After flaming the whole sample was ground and inoculated into 100 ml. Selenite F in a similar manner to the other tissues.

Meal samples

Approximately 30 g. samples were shaken with 100 ml. distilled water for 15 min. The meal was allowed to sediment and the supernatant decanted into a pot containing 100 ml. double strength Selenite F broth.

Rodents and sparrows

The cadavers of rodents and house sparrows were flamed externally and the abdomen opened with sterile instruments. The entire abdominal and thoracic contents were removed, ground with sand and transferred to appropriate amounts of Selenite F broth.

Cultural procedures

The 10 ml. Selenite F broths were incubated for 18–24 hr. at 37° C. whereas the 100 ml. and larger volumes were normally incubated for a full 24 hr. before sub-culturing to deoxycholate citrate agar (Oxoid CM 35) or to 0·5 % sucrose deoxycholate citrate agar (modified Oxoid CM 227). Plates were incubated for 18 hr. and representative non-lactose fermenting colonies were sub-cultured to MacConkey agar (Oxoid CM 7). Colonies on MacConkey agar were subjected to slide agglutination with Salmonella 'O' and 'H' antisera* and to such other standard identification procedures as were necessary. Colonies giving the typical agglutination reaction (O 9+; H gp+, H mt−) were tested in Kohn two tube media (Oxoid CM 179 and CM 181) and those giving the correct biochemical reactions were accepted as *S. dublin*. Representative strains were all confirmed as *S. dublin* by the Central Public Health Laboratory.

* Wellcome Reagents Ltd.

Macro-colony examination

Cultures for macro-colony examination were stored in screw-capped nutrient agar slopes in the dark, at room temperature. Macro-colonies were cultured using modification of the methods suggested by Jameson (1966). Inocula taken from 18 to 24 hr. peptone water cultures were placed on the agar surface by means of a 2 mm. diameter loop. The loop was fashioned from two turns of 24 SWG Nichrome wire one upon the other, with the plane of the loop at right angles to the stem of the inoculating wire. Nineteen colonies were equidistantly inoculated on to each 85 mm. diameter plate, the site of inoculation being determined by a standard placed underneath the agar plate. Macro-colonies were grown on a number of different media incubated at various temperatures; differentiation appeared most satisfactory on two of the media incubated at 32° C. for 3–4 days. These media contained proteose peptone (Oxoid L 46) 2·4 g.; triphenyl tetrazolium chloride 0·004 g.; Teepol 610 (B.D.H.) 0·2 ml.; de-ionized distilled water 200 ml. and either Noble agar (Difco) or Ion agar No. 2 (Oxoid L12) 2·4 g.

When two strains were being compared this was always done on the same plate.

Biotypes of S. dublin

The biochemical reactions of the strains isolated during the investigations were examined in arabinose, dulcitol, xylose and rhamnose and in Stern's glycerol medium (Hall & Taylor, 1970). Single colonies of the isolate to be examined were inoculated into peptone water and incubated for 8 hr. at 37° C.; one drop of this culture was then inoculated into the sugar media. Stern's glycerol media was inoculated from 18 to 24 hr. cultures on solid media.

Control measures

Disinfection

Buildings. Potentially infected faeces and bedding material from the courts, calf-houses, and isolation byres were, with appropriate precautions, removed prior to cleansing and disinfection. This material was ploughed into arable land either immediately or after stacking in the centre of a midden of uninfected manure.

After removal of the bedding the potentially contaminated areas of the accommodation were soaked overnight with a cresol-type approved disinfectant solution. The area was then scrubbed with detergent solution* and washed. Finally, formalin fumigation wherever possible or alternatively a re-spraying with the disinfectant solution completed the operation.

The cleansing and disinfection was carried out by the farm staff as circumstances demanded while excretors were still being detected. In May 1970, when all cattle went out to grass, all cattle buildings were cleansed and disinfected. Throughout the period of observation foot baths containing cresol-type disinfectants were used at the entrances to the various sections of the animal housing by the farm staff and visitors.

Animals. When they had ceased excreting, and immediately before the animals

* By-prox (British Petroleum Ltd).

rejoined their appropriate group within the herd, the hind quarters and tails of infected calves and cows were washed with detergent solution and rinsed with chloroxylenol solution.*

Movements of stock

Non-essential movements of stock were eliminated. Two weeks after infection was first detected the movement of cows between court *J* and the byre *D* ceased: only dry cows thereafter occupied court *J* (Fig. 1).

Recording

Written daily records of all events were maintained throughout the investigation.

RESULTS

The epidemiologically relevant events immediately preceding the onset of the 1969 outbreak are confused and we are still in some doubt as to their relative significance.

Events seemed to be initiated by a cow (N21) calving in court *J* (Fig. 1) on 20 November 1969. The calf was taken to the calf house *E* and on 22 November became ill showing pyrexia and respiratory symptoms. The following day in court *G* a 2-month-old calf (U24) which had come from the calf house on 21 November, was found to be pyrexic and exhibiting symptoms compatible with a mechanical injury to the locomotor system. This animal was immediately segregated from the remainder of the group. On 28 November it developed pneumonic symptoms and diarrhoea, and was found to be excreting *S. dublin*. Shortly after this most of the young calves in calf house *E* showed symptoms and on 1 December, 7 of the 13 calves were found to be excreting the organism. A possible human contact existed between the immediately postparturient cow, N21, and a known *S. dublin* outbreak in calves on an unrelated premises (*T*) some two miles distant; this contact will be mentioned later.

The disease in young calves

The disease in calves less than 3 months of age was characterized by pyrexia, respiratory symptoms, and diarrhoea. In older calves infection may have been associated with transient anorexia. These symptoms were similar to those described by Craig *et al.* (1941).

At the commencement of the investigation calf-house *E* contained 13 calves, ranging from a few days of age to about 3 months, housed in double pens. All calves were treated with chloramphenicol at 150 mg./lb. body weight intramuscularly for 3 days, and 4 days later moved to clean accommodation in a converted byre *C*. This was to remove the known excreting calves from the proximity of the majority of the herd, and to enable the calf-house to be washed and disinfected to receive new-born calves. Fifteen days after moving the calves, clinical symptoms occurred in 5 of them which were then treated with ampicillin

* Dettol (Reckitt and Colman).

(100 mg./30 lb. body weight); temperature and faecal samples taken previously suggested that 3 of these calves had new infections and were not relapsed cases.

Two young calves were destroyed because of their clinical condition, one (N21's calf) on 2 December 1969 and the other on 8 January 1970. Ultimately all 13 calves were demonstrated to have been infected at some time, and faecal excretion by the group persisted for 53 days after the initial discovery of infection in the calf-house.

The drain from the calf-house *C* was sampled at a trap immediately outside the building during the period when it held infected calves. Calf effluent soaked into the straw bedding and percolated into the drains undiluted by water. Positive drain swabs were obtained during the period of faecal excretion and also on the day following the last known excretion by a calf. The subsequent drain swab taken 8 days later proved negative. The calf pens were then cleaned and disinfected: thereafter all faecal and drain swabs proved negative, including a group sample taken two days after castration and de-horning on 13 April 1970. These results are summarized in Table 1.

Faecal excretion in older calves

At the start of the outbreak, courts *F*, *G* and *H* (Fig. 1) held 13 (5–11 months old), 11 (3–5 months old) and 13 (12–18 months old) calves and young stock respectively. Examination of faecal samples from court *F* revealed two animals U10 and U16 excreting between 10 and 29 December. One further group sample (containing animals U4 to U9) proved positive on one occasion (15 December) but an individual excretor could not be identified. Six samples taken between January and April failed to demonstrate further excretion by animals in court *F*.

Court *G* contained the animal U24 which had been isolated because of possible injury at the time when it was found to be infected. This animal was destroyed on 2 December. We were unable to detect any further faecal excretors in either courts *G* or *H* when each group of animals was sampled on six occasions.

Faecal excretion by adult cows

The whole herd was sampled on ten occasions between 3 December and 5 February; thereafter group faecal samples were examined monthly and additional individual samples taken when necessary.

When infection was first detected the majority of the cows were tied up in their own stalls in the byre, except for two periods in the week when they were put out in small groups into an exercising yard immediately outside the byre. As there were more cows than the byre could accommodate, 20 cows shared accommodation, alternating between 10 stalls in the byre and court *J*. This arrangement was terminated on 12 December, from which date only dry cows were housed in court *J*.

During the 12 months following the discovery of infection 8 excreting cows were identified. Five of the 8 cows belonged to the group which shared housing in the byre and court *J* (Fig. 1). Faecal excretion was not associated with observable clinical symptoms in any of the adult cows.

Table 1. *Faecal excretion of* Salmonella dublin *by calves, and related events*

	December								January										February		
Calf or sample	1	2	†	15	18	22	23	29	5	8	12	19	22	23	26	27	28*	29	4	19	23
Calf U17	**0**	.	.	**0**	.	**0**	.	**0**	**0**	D	.	.	.	.	.	.	.	.	.	.	.
U21	**0**	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
U28	**0**	.	.	**0**	.	0	.	0	0	.	0	0	.	.	0	.	.	0	0	.	0[1]
U29	**0**	.	.	0	.	0	.	0	0	.	0	0	.	.	0	.	.	0	0	.	0[1]
U30	**0**	.	.	0	.	0	.	0	0	.	0	0	.	.	0	.	.	0	0	.	0[1]
U33	0	.	.	**0**	.	0	.	0	**0**	.	0	0	.	.	0	.	.	0	0	.	0[1]
U34	**0**	.	.	**0**	.	**0**	.	**0**	0	.	0	0	0	0	0	.	.	0	0	.	0[1]
U35	**0**	.	.	**0**	.	**0**	.	**0**	0	.	0	**0**	**0**	0	0	.	.	0	0	.	0[1]
U36	0	.	.	0	.	**0**	0	0	0	.	0	0	.	.	0	.	.	0	0	.	0[1]
U37	0	.	.	0	.	**0**	.	**0**	0	.	0	0	.	.	0	.	.	0	0	.	0[1]
U38	0	.	.	0	.	**0**	**0**	**0**	0	.	0	0	.	.	0	.	.	0	0	.	0[1]
U39	0	.	.	0	.	**0**	.	0	0	.	0	0	.	.	0	.	.	0	0	.	0[1]
U40	0	.	.	**0**	.	0	0	0	0	.	0	0	.	.	0	.	.	0	0	.	0[1]
Drain swab	.	.	.	.	**0**	.	**0**	.	**0**	.	**0**	.	**0**	.	.	0	0	.	**0**	0	.
Sparrows	.	.	.	.	.	.	.	.	.	.	0	0	.	.	.	.	.	.	.	.	.
Sparrow droppings	.	.	.	.	.	.	.	.	.	**0**	**0**	.	0	.	.	.	.	.	.	.	.

D Killed.

* Pen *C* cleaned and disinfected.

† All calves treated Chloramphenicol 2–4 December; moved to calf house *C* on 8 December.

0 Sampled, positive for *S. dublin*. 0, Sampled, negative for *S. dublin*. 0[1], Also negative on 20 March, 15 April, 4 May and 21 October.

Table 2. *Excretion of* Salmonella dublin *by individual cows*

Cow	December													January									February											
	3	5	9	10	11	12	15	16	17	18	19	22	29	5	6	7	12	14	19	24	26	27	30	2	4	5	6	7	9	11	12	15	17	19	23
	0+	.	.	.	.	.	.	.	**0**+	.	.	.	.	.	.	.	.	.	.	.	**0**+	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R20	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	.	.	0	.	.	.	0	.	.	.	.	0	.	.	.	.	.	.	.	.	0
S17	0	**0**	0	0	0	**0**	0	0	0	0	0	0	0	0	.	.	0	.	.	.	0	.	.	.	.	0	.	.	.	.	.	.	.	.	0
P3	?	0	.	**0**	.	0	0	0	0	0	0	0	0	0	C	0	0	.	.	.	0	.	.	.	.	0	.	.	.	.	.	.	.	.	0
S12	0	0	.	**0**	.	**0**	0	0	0	0	0	0	0	0	.	.	0	.	.	.	0	.	.	.	.	0	.	.	.	.	.	.	.	.	0
S18	0	0	.	0	.	.	0	.	?	.	.	**0**	**0**	0	.	0	0	0	0	.	0	.	.	.	.	0	.	.	.	.	.	.	.	.	0
R28	0	0	.	0	.	.	0	.	.	.	.	.	0	0	.	.	0	.	.	C	?	0	**0**	0	0	0	0	.	0	0	.	.	.	.	0
S34	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	0	.	C	**0**	**0**	0	0	0	0	0
G17	?	0	.	0	.	.	0	.	0	.	.	.	0	0	.	.	0	.	.	.	0	.	.	.	.	0	.	.	.	0	.	.	.	.	0

Cow	June														July													
	2	2	6	8	10	14	15	17	18	19	20	22	23	24	25	26	27	29	30	1	2	3	4	5	6	7	8	
G17	C	**0**	0	0	**0**	**0**	**0**	**0**	**0**	0	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	and 21 further negative samples up to 30 July when slaughtered

C, Calving date.
0, Sampled negative.
0, Sampled positive.
0+, Positive group sample, individual animal not identified.
?, Animal included in positive group sample.

When infection was detected in a faecal sample the individual cow was placed in isolation in the small byre *B* until at least 5 clear samples were obtained after which the animal was returned to the herd. Despite the removal of excreting animals from the herd, new cases continued to be encountered and, in an effort to control the spread of infection, the bi-weekly exercising period was stopped from 12 December. Isolation of individual adult excretors was discontinued at this time.

An additional cow (S18) excreted between 17 and 29 December followed by a period when no further excretion was detected, until cow R28 was found to be infected on 26 January. This animal had been introduced into the byre on 12 January after having given 6 previous negative samples. She calved on 24 January and 2 days later a group sample indicated infection which was confirmed by an individual sample 4 days later. A further animal (S34) introduced into the byre on 26 January, gave a negative sample on 5 February, calved on 7 February and excreted on 9 and 11 February.

Since we were unable to account for a source of infection for either of these animals (the last known previous excretion being detected in calves on 22 January) an extensive examination of the cow stalls was made on 16 February. *S. dublin* was isolated from scrapings taken from two of the stalls at the bottom of the byre which had been used by the court cows, but currently was occupied by cows G17 and S34. The organism was not recovered from R28's stall and neither of the cows previously standing in R28 or S34's stalls could be incriminated as infected either on serological grounds (Lawson *et al.* 1974), or by the detection of faecal excretion. Following this, all the byre stalls were thoroughly cleaned and disinfected. The detection of excretion in S34 and R28 suggested a possible relation between calving and excretion, and from this time all calving animals were sampled daily for at least 7 days following the calving. No further excretion was detected until the cow G17 proved positive on 2 June. This cow had been sampled 13 times between 3 December and 21 April, being negative on each occasion except for a positive group sample taken on 3 December which included faeces from this animal. The excretor was not detected by individual samples taken two days later. Excretion by G17 was detected intermittently between 2 June, the day she calved, and 6 July; 10 of the 25 samples taken during this period proved positive. Twenty-four further daily 20 g. samples were negative prior to her slaughter on 30 July.

During previous sampling of infected cows we had been of the opinion that 2 g. faecal samples were sometimes insufficient to detect excretion. However, in no case had excretion continued long enough to allow a quantitative estimation to be made. From the cow G17 we were able to examine 17 faecal samples, using 20 g. and 2 g. portions from the same sample. *S. dublin* was not identified in any of the 2 g. inoculations but was isolated from 5 of the 20 g. portions of faeces. Faecal excretion among these adult cows is summarized in Table 2.

Drain swabs were taken on a limited number of occasions and at least 3 of these were in appropriate positions when some cows were known to be excreting. None of these or any other swabs from drains containing effluent from adult cows proved positive.

Faecal sampling of all the adult milking animals in the herd continued on a

Table 3. Salmonella dublin *status of yearlings and calves (during December* 1969 *to February* 1970) *and the time of their slaughter or first calving*

	Year of slaughter or first calving		
Month	1970	1971	1972
January	—	—	—
February	—	**0** 0 0	**0**[c] **0**[c]
March	—	**0** 0 0	*0* 0 **0**[c] 0[c]
April	—	**0** 0 0 0	—
May	*0 0 0* 0 0 0 0 0	**0*** **0** 0 0	—
June	—	**0 0**	
July	—	—	—
August	*0* 0 0	—	—
September	—	**0**[c] *0*[c] 0[c] 0[c] 0[c] 0[c]	—
October	*0 0* 0 0	0[c]	—
November	0	0	—
December	0	—	**0**

Animals slaughtered	Animals sampled at first calving	Status (December 1969–February 1970)
0	**0**[c]	Known *S. dublin* excretors
0	*0*[c]	Possible serological evidence of infection
0	0[c]	No known excretion or serological evidence of infection

0* Animal U38 – positive culture *S. dublin* from bile at slaughter

monthly basis until January of 1971, a year after the discovery of infection. Parturient cows continued to be sampled as described previously until March 1971, by which time all had calved once since the appearance of herd infection. In addition the heifers which had been infected as neonates were similarly examined at the time of their first calving between September 1971 and February 1972. (Table 3).

Miscellaneous investigations

Milk

Examination of group milk samples from the herd taken on 5 December 1969 failed to demonstrate the presence of *S. dublin* and no further milk samples were taken.

Placentas

Placentas from 17 animals were obtained during the period June 1970 to March 1971. In 10 instances the placentas came from potentially infected animals at their first parturition following herd infection; these included 5 known faecal excretors and 5 with possible serological evidence of infection. In no case was *S. dublin* isolated from a placenta.

Other domestic livestock

Faeces from all the housed sheep, farm dogs and representative samples from poultry were examined at the outset and proved negative. The hysterectomy-derived piggery (*K*) contained the only other livestock in close proximity to the main cattle herd and these were not thought to be at risk owing to the isolation

procedures in operation. On the 9 February 1970 an 8-month-old pig died with gross lesions of lymphosarcoma, and a fibrinous enteritis. *S. dublin* was isolated from the small intestine of this pig and from a further litter-mate also affected with lymphosarcoma in which the organism was recovered from pneumonic lesions at necropsy on 21 May. Initially, the origin of these infections was inexplicable until further investigation revealed a possible explanation. The drains from the byre, calf-house and piggery join a common main drain which is liable to occasional blockage. When the main drain becomes blocked, effluent flows back into the piggery drains which are on a lower level than the other buildings; the pen first affected by this back-flooding is the pen which housed the infected pigs and the drain was known to have become blocked during the late autumn of 1969. No further evidence of infection within the piggery was discovered and no other possible methods of infection could be substantiated.

Rodents and sparrows

Though rats (*R. norvegicus*) and mice (*Mus musculus*) were not particularly common on the farm it was possible to examine 16 specimens caught in the area adjoining calf-house *E*. *S. dublin* was not recovered from any of these rodents. Large numbers of house sparrows (*Passer domesticus*) used the infected calf-house *C* for roosting, and their presence constituted a possible method of spread of infection. Sparrow droppings collected from this house on 8 and 12 January yielded *S. dublin*; thereafter samples taken from this site were negative. The viscera of 15 sparrows collected in this house also failed to yield the organism. In addition, large bulked samples of all recognizable faecal droppings collected on 12 January from 5 roosts and feeding perches elsewhere on the farm failed to yield *S. dublin*.

Animal feeds

Representative samples of fish meal, protein concentrate (2 samples), prepared calf feed and milk substitute (2 samples), in use at the commencement of the outbreak were examined for the presence of *S. dublin* with negative results.

Calf accommodation M and N

On 17 January a 4-week-old Friesian calf was introduced to accommodation *M*. A routine faecal sample taken on admission yielded *S. dublin* as did the viscera at necropsy on 19 January. Subsequent to these isolations, mortality due to *S. dublin* took place in young dairy calves in block *N*. We were satisfied however, that there was no connexion between the infections in premises *M* and *N* and the farm outbreak because the macrocolony type of the isolates from the calves in *M* and *N* were identical, but distinct from any of the types derived from the dairy farm (see below).

Examination of tissues from slaughtered animals

This work has not been completed because it is dependent on the culling of animals from the milking herd. To date 30 of the original 55 adult cows present in the herd at the time of infection, together with 21 of the 32 calves and all the 13 yearlings have been examined.

Table 4. Salmonella dublin *status of adult cows (during December* 1969 *to February* 1970) *and the time of their slaughter*

	Year of slaughter		
Month	1970	1971	1972
January	—	00	**0**
February	0	*00*	00
March	—	—	—
April	*0*	*00*	—
May	000	—	*00*
June	—	—	—
July	000[1]	—	—
August	—	*00*[2]	—
September	00	00	—
October	—	—	—
November	—	—	**0**
December	00	00	—

Infected status at December 1969/February 1970:
0 Known faecal excretors *S. dublin*.
0 Possible serological evidence of infection ('O' > $\frac{1}{80}$ or 'H' > $\frac{1}{80}$ or CFT (P) > $\frac{1}{4}$).
0 Not thought to have been infected.
[1] Cow G17 positive *S. dublin* at slaughter (see text).
[2] Cow N21 (see text) and all others negative at slaughter.

Adult cows

Ten cows known to have been faecal excretors or to have had raised serological titres (Lawson *et al.* 1974), have been examined at slaughter. The details of these adults are given in Table 4. *S. dublin* was recovered from the tonsils, and from a 20 g. sample of the intestinal contents of animal G17. This latter material, portions of the spleen (20 g.) and the liver (20 g.), the supramammary lymph nodes and seven portions of the small intestinal wall were examined in addition to the tissues routinely examined. The macrocolony type produced by the isolates obtained at slaughter was identical with those produced by isolates recovered from faecal samples between 2 June and 6 July.

One animal, N21, had shown a more persistent serological titre than any of the other animals and for this reason the following additional tissues were examined: supramammary lymph nodes, mediastinal lymph nodes, gall bladder wall, and small intestinal contents. *S. dublin* was not isolated from any of these tissues. The remaining 19 cows believed to be non-infected failed to yield the organism at slaughter.

Yearlings (court H)

Faecal excretion had not been demonstrated in this group although serological tests indicated that at least 2 animals had been exposed to infection. These animals were slaughtered 5–10 months after possible exposure and proved culturally negative.

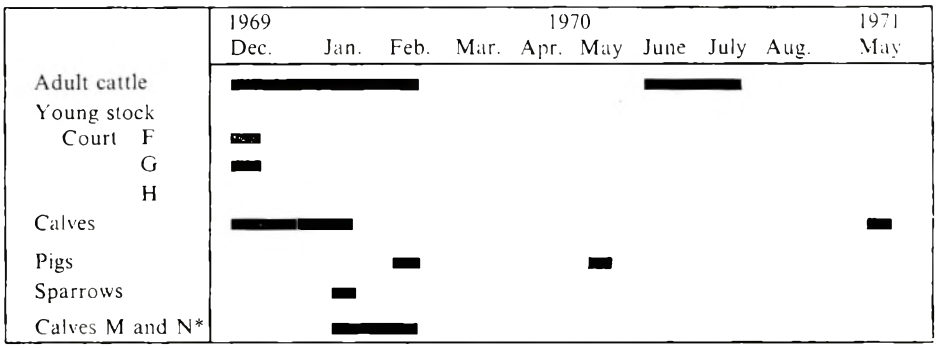

Fig. 2. Summary of known infection in outbreak. * Infection originated from different source.

Calves (courts F and G)

Both courts contained animals shown to have been faecal excretors and serological examination indicated the presence of additional infected animals. Thirteen of the 23 animals were slaughtered 10–22 months after exposure and all proved negative.

Calves (calf-house E)

This group were all known faecal excretors as neonates. Eight of the 10 animals were slaughtered 12–24 months after infection and *S. dublin* was isolated from one (U38). Following neonatal infection, 10 negative samples had been obtained from this animal before the recovery of the organism from the gall bladder at slaughter. Three animals (W5, 8 and 9) thought to be susceptible and not previously exposed to infection, had been introduced into the court occupied by U38 3 days before her slaughter. Faecal examination and culture of the tissues of these animals at slaughter 42 days later failed to yield *S. dublin*, although serological examination of paired serum samples yielded some evidence that they may have been exposed to infection. These results are recorded in Table 3 and the known infections of the whole outbreak are summarized in Fig. 2.

Identity of strains isolated

Macro-colony examination

Macro-colonies grown from cultures isolated from a number of the infected cattle were compared. The macrocolonies proved to have a range of forms and if the whole range of isolates had not been available those at the extremities of the range might have been considered to be dissimilar. We were unable to link any particular colony type with the calves, young stock or adults, each group seeming to contain some of the variations observed. The macrocolony type of *S. dublin* present on the premises *T* which had been a possible source of infection for the outbreak (see Results: History) differed slightly but consistently in appearance from any of the farm isolates. This difference caused us to critically re-examine the events leading up to the discovery of infection and helped us to conclude that it was extremely unlikely the two outbreaks were related.

The majority of macrocolony examinations were carried out on single isolates from individual animals but in the case of cow G17 a series of 10 cultures isolated at different times were examined. All isolates produced identical macrocolonies.

The macrocolonies produced by the two isolates obtained from pigs were similar to each other and did not differ from some of the bovine isolates. The isolate obtained from the bile of animal U38 at slaughter 17 months after neonatal infection differed by being anaerogenic and of different macrocolony type to the isolates from the initial herd infection. In view of the long period between calfhood infection and slaughter we do not feel that these colonial differences preclude the possibility that the isolates had a common origin.

Biotype

Seven out of the 12 isolates examined from the farm outbreak could be classified as belonging to Biotype D. However, altogether 6 biotypes were represented in these isolates; A, B, D, G, T and L. The variability of these results was confirmed by repeated examinations of fermentation reactions in arabinose and Stern's glycerol media using replicate and different batches of media. Acid was produced from arabinose within 14 days by 12 out of the 33 isolates from the farm with a range of from 2 to 19 days (mean 15 days).

DISCUSSION

Investigations into the epidemiology of *S. dublin* infection in cattle have largely concentrated on studies of clinical infection or of localization in the gall bladder in known excretor animals (Field, 1948; Gibson, 1958). The majority of these studies have taken place on cattle from areas where fascioliasis is endemic. This intercurrent disease which affects the localization of the organism, in the tissues of the bovine (Frik, 1969) does not occur in the herd we investigated. As Watson, Wood & Richardson (1971) point out, no attempt has been made to examine the tissues of non-excreting animals in known infected herds. In the herd studied by these authors the results obtained from the slaughtered animals suggested that in some cases at least the animals were undergoing recent infection, and their results resembled those found in animals slaughtered after some days of travel (O'Conner, Murphy & Timoney, 1967). For this reason their results give little information on the possible persistence of the organism in the tissues of recovered animals. One difficulty in examining bovine tissues is the volume of material which may potentially harbour the organism and the obvious impracticability of examining more than a small proportion of this tissue. We are aware of this shortcoming in our own study and it was with this in mind that the lymphoid tissues examined were chosen.

The herd studied in this report was exceptional in the facilities for repeated sampling and for the handling of animals prior to slaughter.

The investigation described gives some indication of the problems which might be encountered in trying to eliminate *S. dublin* from a cattle herd and also provides a rational explanation for the appearance of clinical infection in young animals

in the absence of the introduction of infection to the herd or the apparent absence of known carrier animals. Two carrier animals were detected and one of these (G17) was a young animal in the herd at the time of the previous clinical infection in 1963 and may have been infected since that time. At slaughter *S. dublin* was isolated from the tonsils which may have been shedding organisms into the alimentary canal at a level which had not been detectable by faecal sampling during the previous 24 days. Persistent tonsillar infection has been demonstrated in sheep (Gitter & Sojka, 1970) although in the instance cited it was not associated with persistent faecal excretion. The demonstration of *S. dublin* in the gall bladder of U38, 17 months after infection, indicates a further potential hazard in any attempt to eliminate *S. dublin* from a herd. Although we have no estimate of the number of organisms present in the bile, the repeated negative faecal samples from this animal (U38) following calfhood infection may indicate that only small numbers of organisms were present in the gall bladder. This finding is contrary to the observations of others who have regularly associated gall bladder infection with detectable faecal excretion (Gibson, 1958; Frik, 1969).

In three instances excretion by adult cows was closely associated with parturition, and in cow G17 the evidence suggests that this may have been a quantitative increase rather than an absolute change in excretor status. In 1 of the other 2 cows (R28) the serum titres indicated that infection had taken place some 2 months prior to excretion and parturition. These observations demonstrate a mechanism for the previously postulated link between calving and the appearance of clinical herd infection (Report, 1965). This parturition-associated excretion may be restricted to a period following infection because the examination of the remainder of the herd at parturition over the next year failed to disclose further excretors.

There was a significant difference in the incidence of infection between varying ages of calves. All the neonate calves rapidly became infected following exposure whilst the older calves in court *A* varied in response. These latter animals were all 'loose-housed' together but despite the presence of at least 3 excretor animals other members of the group did not become infected as judged by the absence of serological responses and failure to detect faecal excretion. In other circumstances without this evidence it might have been assumed that all the group had been infected.

Rats and mice have been incriminated as potential carriers of infection (Gibson, 1958). However, house sparrows probably constitute at least as great a potential threat in the spread of infection. In the autumn, flocks of this species disperse locally over a radius of a few miles (Summers-Smith,1963). Our evidence suggests that this species is unlikely to be commonly involved in the spread of infection either between or within infected farms.

Simple and easily applied methods for detecting infected herds or premises are of great practical value. Robinson (1966) suggested that drain swabs were useful for detecting *S. typhimurium* infection among adult cattle. In our investigations drain swabs provided an accurate assessment of the *S. dublin* excretor status among calves but we have no confidence in their use for detecting

low levels of excretion by adult cattle infected with this serotype when the animals are housed in byres.

Macrocolonies, like most epidemiological markers, did not provide an absolute guide to the identity of different isolates thought to originate from the same source. However, they were most useful in the demonstration of identity between the isolates obtained from G17 before and at slaughter. In the absence of this information the isolates from the tonsil and intestine might have been considered to have originated from infection in the lairage. Our experience in biotyping the isolates obtained in this outbreak would indicate that, where carbohydrate fermentation is mutative (Kauffman, 1954), the mutation rate is likely to be so variable that some of the biotypes of Hall & Taylor (1970) do not have epidemiological significance.

The control of spread of *S. dublin* infection amongst housed adult cattle can be achieved by simply restricting the movement of animals, whilst infection in calves can be controlled by isolation and disinfection. The detection of the carrier animal still remains a problem and failure to identify these animals may result in the re-appearance of infection at a later date.

This study would not have been possible without the whole-hearted co-operation of a large number of people; in particular, we would like to acknowledge the valuable assistance of the Veterinary Staff of Edinburgh Corporation abattoir and the Staff of the involved farm.

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The epidemiology of *Salmonella dublin* infection in a dairy herd

II. Serology

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SUMMARY

A number of serological tests were evaluated in a study of *Salmonella dublin* infection in a dairy herd. None of the tests used detected either of the two carrier animals from which *Salmonella dublin* was isolated at slaughter 7 and 17 months after the herd infection. The complement fixation tests used proved to be a better guide to the presence of recent herd infection than the conventional 'O' or 'H' agglutination tests.

INTRODUCTION

Serological tests have been employed during studies of bovine salmonellosis both in the field disease (Field, 1948) and in experimentally infected cattle (Frik, 1969). These workers employed conventional salmonella 'O' and 'H' antigens. The passive haemagglutination test has been utilized in studies on salmonellosis of other species (Seiburth, 1957) and although this test differs in sensitivity it appears to measure a similar antibody to that determined by conventional 'O' agglutination tests. The serological responses of cattle to infection by *Brucella abortus* as measured by the complement fixation test (Jones, Hendricks & Berman, 1963) or the Coombs test (Cunningham, 1968) sometimes differ from the response measured by the agglutination titre (Wisniowski, 1964), and are therefore additionally a better indication of the time of the infection.

The following is an account of the serological responses encountered in a herd of cattle infected with *Salmonella dublin* and the relationship between these results and the recovery of the organism during life and at slaughter. The details of the detection of infected cattle and the circumstances of the outbreak have been discussed in a previous paper (Lawson, McPherson, Laing & Wooding, 1974).

MATERIALS AND METHODS

Sera

Blood samples from the jugular vein of calves or the coccygeal vein of adult cattle were taken into vacutainer tubes.* Serum was obtained from these samples after clotting, using centrifugation when necessary, and stored at $-20°$ C.

Serological tests

'H' antigen. Nutrient agar slopes were inoculated with an eighteen hour peptone water culture of *S. dublin* (1420/69). After incubation for 18 hr. at 37° C the growth

* Becton Dickinson.

was washed off with 0·2% formol saline. This concentrated antigen suspension was stored at 8° C. and diluted with normal saline before use. Batches of antigen were standardized by titration with a positive bovine *S. dublin* serum in a similar manner to the procedure employed for *Br. abortus* antigen (Alton & Jones, 1967), the density of the diluted antigen corresponding approximately to Brown's opacity tube 3. *S. dublin* antisera used for standardization were kept in small aliquots at −70° C.

'H' titres were read by recording the amount of deposited agglutinated antigen. Some difficulty was experienced in accurately estimating the end-point as there was a tendency for some sera to produce slight agglutination over two and occasionally more dilutions. For this reason the test result was recorded as the last dilution giving 50% or more agglutination. In later tests a quantitative control of this end point was included for comparison; this utilized a known positive serum to which a half unit of antigen had been added.

'O' antigen. A non-motile strain of *S. dublin* (322/68) was grown on nutrient agar slopes using the method employed for the production of 'H' antigen. The growth was washed off the slopes with the minimum amount of normal saline and 20 volumes of absolute alcohol added to one volume of antigen suspension. The mixture was held at 50° C. in a water bath for half an hour after which the antigen was washed twice in normal saline by centrifugation and re-suspended in 0·25% formol saline. Dilution and standardization were as for the 'H' antigen.

Complement fixation test antigens. The two antigens employed were the alcohol treated 'O' antigen (CFT(A)) suitably diluted in veronal buffer and a phenolized suspension (CFT(P)) prepared from the non-motile 322/68 strain in a similar manner to that employed in the production of *Br. abortus* agglutinable suspension (Alton & Jones, 1967).

Agglutination tests

Doubling dilutions of antisera were prepared in 75 × 10 mm tubes and equal volumes of antigen added to the diluted sera to make a final volume of 1·0 ml. 'H' agglutination tests were incubated at 37° C. for 2 hr., allowed to stand overnight at room temperature and then recorded as the amount of agglutinated antigen deposited at the bottom of the tube. 'O' agglutination tests were incubated overnight at 37° C. and the density of the supernatant antigen compared with similarly incubated standards containing 1, $\frac{3}{4}$, $\frac{1}{2}$ and $\frac{1}{4}$ units of antigen suspended in saline.

Coombs tests

'O' agglutination tubes showing no agglutination were centrifuged to deposit the antigen which was then washed three times in 1 ml. volumes of normal saline and finally resuspended in 0·4 ml. rabbit anti-bovine serum optimally diluted with normal saline. The tubes were re-incubated overnight at 37° C. and the test read as the amount of deposited antigen. The rabbit anti-bovine serum did not agglutinate *S. dublin* 'O' antigen in tube agglutination tests at a dilution of 1/2, and in the Coombs test it was used at a dilution of 1/80.

Complement fixation tests

Sera to be tested were heated at 56° C. for ½ hr. All tests were carried out in WHO Perspex trays using veronal buffer (Oxoid BR16) as diluent. Defibrinated washed sheep cells at 2 % and equal volumes of suitably diluted haemolytic serum* in buffer (containing 2 units haemolysin) comprised the haemolytic system.

After preliminary complement titration, antigens were titrated in the presence of excess complement using the serum from an animal known to have been infected by *S. dublin*. The quantity of antigen chosen as a result of this titration was that amount which gave the highest titre with the positive serum. In the test itself sera were serially diluted in 0·2 ml. volumes of buffer and to these were added one volume (0·2 ml.) of diluted antigen and one volume of diluted guinea-pig complement (containing 2 MHD_{100} complement). After overnight fixation at 5° C. the test trays were warmed at 37° C. for five min. and one volume of haemolytic system added. The trays were agitated to mix and then incubated at 37° C. for 1 hr. A range of control dilutions prepared without antigen was always included for each serum, and sera showing anti-complementary activity in the range of a positive result in the test itself were discounted. A positive control bovine serum, stored in small volumes at −70° C. was titrated with each series of tests.

RESULTS

It is known that sera from cattle thought not to have been exposed to infection with *S. dublin* show titres against both the 'O' and 'H' antigens of this organism (Field, 1948). Presumably these antibodies are induced by exposure of the animals to other members of the enterobacteriaceae or other bacteria which possess related antigens.

For comparative purposes the serum titres found in a herd of cattle on a neighbouring farm from which routine necropsies and cultural examination of clinical material over a period of many years failed to demonstrate any evidence of infection with *S. dublin* are listed in Table 1.

Adult cattle

The serum titres found in the herd at the time of active infection are given in Table 2. Where faecal excretion of *S. dublin* was detected from individual animals the titres obtained subsequent to the discovery of infection are given. The later results obtained after the period in which infection was demonstrated in adult animals are given in Tables 3*a* and 3*b*. In all the tests carried out during the period of active infection, a high proportion of known infected animals showed titres in the upper range. In every test, however, a varying number of animals showed raised titres which did not correlate with known infection. The distribution of serum titres found in the 'O', 'H', Coombs and CFT(A) tests approximated to a normal distribution about the mean. However, in the case of the CFT(P) the test tended to divide the animals into two distinct groups although the lower group still contained known infected animals. The CFT employing alcohol antigen

* Haemolytic serum (horse), Wellcome Reagents.

Table 1. *Distribution of serum titres (reciprocal) against* Salmonella dublin *in 29 adult cattle in an uninfected herd*

Agglutination test			CF test (phenol antigen)	
	'O' antigen	'H' antigen		
Titre	No. of animals	No. of animals	Titre	No. of animals
10	0	7	< 4	28
20	0	13	4	1
40	18	9	8	0
80	11	0	16	0
Mean titre	55·0	24·0	0·138	

(CFT(A)) proved more sensitive and yielded higher titres with the sera from known infected animals than the test carried out with the phenol antigen (CFT(P)). This increased sensitivity avoided some of the problems associated with the interpretation of result from sera which were anticomplementary. Later experience, however, indicates possible disadvantages in the use of the test employing an alcohol antigen (see below). In each of the four tests ('O', 'H', Coombs and CFT(A)) many titres fell within the range where some doubt might exist as to the interpretation of the result.

Some six months after the period of active infection the mean 'O' and 'H' titres had varied little from the previous tests (Table 3*a*) although in the case of the 'O' agglutination tests the number of animals with titres of 1/320 and above had fallen considerably. The most dramatic reduction in the mean herd titre occurred with the CFT(P), where of eleven animals previously showing high titres all but one (a known infected animal) had returned to 1/8 or less. By this time the titres in the CFT(A) had fallen more markedly than in the 'O' and 'H' tests but less than the titres in the CFT(P).

Fifteen months after infection the whole herd showed an increase in the level of 'O' and 'H' titres, not associated with known infection, and the majority of sera gave 'O' titres of more than 1/80. The CFT(A) showed a number of animals with newly acquired titres of 1/32 or more which might have been considered as indicative of infection but these titres could not be related to known past or present infection. The CFT(P) on these animals with raised CFT(A) titres gave levels of less than 1/8 in every case except animal N21 which retained her raised titres until slaughter (CFT(A) 1/32; CFT(P) 1/16).

Of the adult animals exposed to infection and subsequently slaughtered, only one animal (G17) yielded *S. dublin* and a comparison between this animal and those negative at slaughter is shown in Table 4. All these animals, except two not thought to have been infected were slaughtered more than 4 months after the period of active infection.

Table 2. *Serum titres in adult cattle, infected farm December* 1969–*January* 1970 (*infected phase*)

'O'		'H'		Coombs		(CFT(P)		CFT(A)	
Titre	No.	Titre	No.	Titre	No.	Titre	No.	Titre	No.
10	1	< 10	7	40	5	< 4	27	< 8	5
20	8	10	15	80	10 (1)	4	10 (2)	8	3
40	6	20	15 (3)	160	13 (1)	8	0	16	17 (1)
80	23 (3)	40	4 (2)	320	7 (1)	16	7 (3)	32	13 (1)
160	1 (1)	80	4 (1)	640	2 (2)	32	2	64	4 (2)
320	7 (1)	> 80	2 (1)	1280	2 (2)	> 32	2 (2)	> 64	5 (3)
640	1 (1)	–	–	> 1280	1	–	–	–	–
1280	1 (1)	–	–	–	–	–	–	–	–
Mean titre	80		26		–		7·15		34

Numbers in parentheses denote number of animals known to have excreted *S. dublin*.

Table 3*a*. *Serum titres in adult cattle, infected farm June* 1970 (6 *months after infection*)

'O'		'H'		CFT (P)		CFT (A)	
Titre	No.	Titre	No.	Titre	No.	Titre	No.
10	0	< 10	9 (1)	< 4	29 (5)	< 8	3
20	3	10	12 (3)	4	9 (1)	8	4 (1)
40	9 (1)	20	12 (1)	8	4	16	22 (3)
80	24 (4)	40	6 (1)	16	1 (1)	32	11 (2)
160	4 (1)	80	3 (1)	32	0	64	3 (1)
320	2 (1)	> 80	1	> 32	0	> 64	0
640	1	—	—	—	—	—	—
Mean titre	80		23		1·95		21·6

Table 3*b*. *Serum titres in adult cattle, infected farm March* 1971 (15 *months after infection*)

'O'		'H'		CFT (P)*		CFT (A)	
Titre	No.	Titre	No.	Titre	No.	Titre	No.
10	0	< 10	1	< 4	4	< 8	7
20	0	10	7 (1)	4	0	8	12 (2)
40	3 (1)	20	12 (2)	8	1	16	8 (2)
80	10 (1)	40	12 (3)	16	0	32	5 (1)
> 80	23 (5)	80	2 (1)	32	0	64	4 (1)
		> 80	2	—	—	> 64	0
Mean titre	128		35		—		18

* CFT (P) only carried out on those animals in which CFT (A) inexplicably raised.

Table 4. *Serum titres of cows at slaughter present in herd as adults during infection*

'O'		'H'		CFT(P)		CFT(A)	
Titre	No.	Titre	No.	Titre	No.	Titre	No.
20	1	10	8	< 4	13	< 4	12
40	2	20	10	4	1	4	4
80	11	80	0	8	0	8	5
160	9	160	2	16	1	16	0
—	—	320	1	—	—	32	1
—	—	—	—	—	—	64	1
Titres of cow G17, tonsillar infection present at slaughter							
160		80		< 4		16	

Young cattle (*court H*)

In this group of 13 fattening cattle, faecal excretion was not detected on the six occasions that the animals were sampled, though infection was present in the animals on either side of this court. The divisions between the courts were of simple tubular construction. Serological testing during March 1970 yielded the results in Table 5.

Table 5. *Young cattle court H* (*March* 1970)

'O'		'H'		CFT(P)		CFT(A)	
Titre	No.	Titre	No.	Titre	No.	Titre	No.
< 10	0	< 10	1	< 4	11	4	0
10	0	10	4	4	0	8	1
20	0	20	6	8	1	16	2
40	0	40	1	16	1	32	5
80	9	80	1	32	0	64	5
> 80	4	> 80	0	—	—	—	—
Mean	105		21·5		1·9		40

Table 6. *Serum titres young cattle court F* (*March* 1970)

'O'		'H'		CFT(P)		CFT(A)	
Titre	No.	Titre	No.	Titre	No.	Titre	No.
< 10	0	< 10	1	< 4	6	8	0
10	0	10	8	4	0	16	1
20	0	20	0	8	3	32	8
40	4	40	1 (1)	16	1	64	0
80	7 (1)	80	3 (1)	32	0	> 64	4 (2)
> 80	2 (1)	> 80	0	64	3 (2)	—	—

Figures in parentheses refer to known infected animals.

All these animals were slaughtered between 5 and 10 months after infection was first identified in the herd, and in no case was the organism recovered from the tissues. At the time of slaughter the mean reciprocal titres for these animals were:

'O', 80; 'H', 23; CFT(P), 2·5; CFT(A), 8.

Young cattle (*courts F and G*)

Infection was present in December 1969 and in March 1970 the serum titres of the animals in court *F* were as shown in Table 6.

The table shows the correlation between the presence of known infection and raised CFT titres, this correlation being less confused by inconclusive reactions than in the case of 'O' titres. Among known infected young cattle the 'H' titres were lower than those encountered among infected adults. Nine months after infection the 'O' titres of the two previously known infected animals were still raised above the group level, whilst the CFT results had fallen within the range encountered in non-infected animals.

The animals from courts *F* and *G* were all examined at slaughter or sampled at calving 10–17 months or 19–26 months, respectively, after active infection. The serum titres are shown in Table 7.

Calves infected as neonates (calf-houses *E* and *C*)

The titres recorded in Table 8 were obtained when calves were sampled 5 months after infection. By this time none had raised 'O' agglutination titres and they could not be differentiated on this basis from a normal non-infected group of calves at

Table 7. *Serum titres of occupants of courts F and G 10–26 months post-infection period*

'O'		'H'		*CFT(P)		CFT(A)	
Titre	No.	Titre	No.	Titre	No.	Titre	No.
10	0	< 10	6 (1)	< 4	10 (2)	< 8	12 (1)
20	1	10	5	4	0	8	7 (1)
40	7 (2)	20	5	8	1	16	3
80	12	40	6 (1)	16	1	32	1
> 80	3	80	1	—	—	—	—

* Some animals not tested.

Table 8. *Serum titres of calves May* 1970

(All animals infected 5 months previously.)

'O'		'H'		CFT(P)		CFT(A)	
Titre	No.	Titre	No.	Titre	No.	Titre	No.
< 10	0	< 10	2	< 4	7	< 4	2
10	0	10	3	4	3	4	1
20	2	20	2	8	0	8	4
40	6	40	1	16	0	16	2
80	3	80	1	32	0	32	0
> 80	0	> 80	2	64	1	64	0
						> 64	1

the farm whose 'O' titres ranged from 1/10 to 1/80. In comparison, the range of 'H' titres was markedly raised above that for the normal calves in which the titres were less than 1/10. One animal (U38) had maintained both a high CFT(P) and CFT(A) titre unlike its cohorts in which these tests were both essentially negative.

Ten months after neonatal infection the serum titres of the group fell within the normal range and when sampled at either slaughter or calving, respectively, the titres shown by all the animals fell within the range encountered in the slightly older animals from courts *F* and *G* (Table 6).

As recorded previously *S. dublin* was isolated from the bile of U38 at slaughter (Lawson *et al.* 1974) but not from other individuals of comparable age and similarly infected as neonates. Whilst this calf showed raised CFT(P) and CFT(A) titres persisting for 5 months it could not be differentiated serologically at the time of slaughter from the other members of the group.

DISCUSSION

Serological tests may be employed in a variety of ways in attempting to evaluate the status of animals thought to have been exposed to infection. In salmonellosis of cattle, these tests are likely to be utilized in two main ways: (i) to give some indication of infection within the herd as a whole and, (ii) to indicate those animals in which infection is persisting in order that the threat posed by these carriers may be eliminated.

A serological test has additional epidemiological value if it indicates infected animals for a restricted period of time following infection so that the serological titres can be related to recent events within the herd. The selected test should also indicate infection in a high proportion of infected animals.

Our experience is that the CFT(P) fulfils these requirements and provides a better basis for indicating herd infection in adult cattle than do the other serological tests that have been used previously. In the circumstances of sub-clinical infection in adult cattle it would appear that the CFT(P) generally becomes negative within 6 months of infection. We encountered fluctuations in the mean herd 'O' titres which could not be related to known infection. This observation is in accord with the known gradual acquisition of 'normal' antibody titres observed in young calves as they reach maturity. The CFT(P) titres do not appear to be influenced by the events which bring about these changes in 'O' titres. CFT(A) titres persist longer than do CFT(P) titres and from our results it appears they may be influenced by non-specific factors which stimulate fluctuations in 'O' antibody titres. 'H' titres proved unreliable in detecting proved infection and when raised persisted for a long time.

At slaughter, none of the serological tests identified the two animals in which infection was demonstrated. The persistence of raised CFT(P) or CFT(A) titres beyond the period of active excretion of the organism by calves may give some indication that the organism has not been eliminated from the tissues. With time, however, these titres regress and no longer give an indication of persistent infection. In both the complement fixation tests the amount of complement utilized was kept as low as possible to ascertain if the tests were capable of detecting carrier animals. At this level of complement some difficulty was experienced with anti-complementary sera, and it may be that, as the tests were not capable of detecting carrier animals, a more useful test might utilize a higher dose of complement.

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The recovery of anaerobic bacteria from swabs

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SUMMARY

When a standard sample of a simulated exudate containing known numbers of anaerobic bacteria was taken up on a swab and plated on solid medium, the number of colonies subsequently cultured represented a very small proportion of the original sample. Evidence is produced that the apparent loss is not primarily attributable to inactivation on the swab but rather to retention of organisms on the swab. This was demonstrable with *Clostridium welchii* and with *Bacteroides* species that have hitherto been regarded as relatively oxygen-sensitive.

When stock strains of *Bacteroides* species were held for some hours on swabs, some progressive loss of viability was demonstrable. A measure of protection was afforded when these organisms were held aerobically on blood agar medium, but a very exacting anaerobe and some wild strains of faecal anaerobes showed gradual inactivation under these conditions.

These findings may have important implications in relation to currently employed bacteriological sampling procedures with swabs in clinical practice.

INTRODUCTION

It is generally accepted that, as a routine for the bacteriological sampling of wound exudates and similar material occurring in insufficient amount to be sent as frank pus in a container, serum-coated swabs should be used (Rubbo & Benjamin, 1951; Cruickshank, 1953); steam-sterilized swabs coated with bovine albumin have also been recommended (Bartlett & Hughes, 1969).

The present study was designed to assess the losses that may occur in sampling anaerobic bacteria from an exudate with a swab that is transported under aerobic conditions and is subsequently plated on solid media in the laboratory where the organisms may be subjected to further aerobic exposure pending anaerobic processing. Some of the results seem to be of general relevance to clinical aerobic and anaerobic bacteriology.

MATERIALS AND METHODS

The test strains included *Bacteroides necrophorus* NCTC7155 and 10575; *B. fragilis* NCTC9343; *Clostridium welchii* (*Cl. perfringens*) Lab. nos. L2A and C1; *Cl. tetani* type VI, NCTC9569; *Cl. oedematiens* type D, Lab. no. GR1D; and

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Escherichia coli Lab. no. M15. *Bacteroides*-like organisms isolated in Edinburgh from human wound exudates and faeces were labelled Gram-negative anaerobic bacilli (GNAB) 1–50. The simulated exudate was prepared as follows: A 0·02 ml. drop of a dilution of an overnight cooked meat broth culture of the test organism calculated to give some thousands of bacteria was mixed in a small ovoid area (2 × 1 cm.) with four 0·02 ml. drops of sterile defibrinated horse blood (Wellcome) on a sterile plastic dish surface. The total volume (0·1 ml.) was absorbed on a sterile swab. The actual number of organisms in the diluted broth used for the simulated exudate was subsequently determined by a spread-plate surface viable count procedure.

Commercially available albumen-coated cotton-wool swabs on wooden sticks were used (Exogen Ltd, Dumbarton Road, Glasgow, W.4). These are supplied in plastic tubes; the same product originally labelled 'serum swab' is now labelled 'albu swab'.

Cultures of anaerobes were incubated at 37° C. in anaerobic jars with room-temperature catalysts (Baird and Tatlock Ltd) and a gas mixture of 90% hydrogen and 10% carbon dioxide (Collee, Rutter & Watt, 1971). Blood agar plates contained Columbia Agar Base (Oxoid) with 10% defibrinated horse blood (Wellcome); unless otherwise stated, the plates were used within a day of their preparation to simulate routine laboratory practice. Other media and special technical procedures were as detailed by Watt (1973).

RESULTS

Development of the test model

A known volume of broth containing a known number of organisms was mixed on a sterile surface with 4 volumes of sterile defibrinated horse blood to make a simulated exudate. The mixture (0·1 ml.) was then completely taken up on a sterile 'Exogen' serum-coated swab. In due course, a swab was plated on to half of the surface of a blood agar plate. The swab was rotated during the plating process to give a generous primary seeding of that area of the plate that bacteriologists call the 'well'.

The release of organisms from the swab to solid medium

The test swabs yielded only a small proportion (3–5%) of their total viable load when they were used to prepare 'wells' in the course of seeding plates of blood agar medium. This was so when *Clostridium welchii* or various *Bacteroides* species or *Escherichia coli* were used and the finding was consistently reproducible. For example, swabs loaded with *ca.* 5000–7000 organisms of various *Bacteroides* species yielded *ca.* 100–300 colony-forming units to the plate, and plates seeded from swabs loaded with *ca.* 1500 *Cl. welchii* organisms yielded *ca.* 50 colonies. In the latter case, there was no doubt that vegetative cells were yielding colonies; there were less than 10 spores in the inoculum on each swab.

When the same loaded swab was used to seed a series of plates, there was a fairly reproducible dose given to each plate. Thus, a swab bearing an estimated

Table 1. *The influence of delay and method of processing on the recovery of* Cl. welchii *from test swabs each bearing* 1500 *organisms*

At time (hr.)	Viable counts from duplicate platings of test swabs: Plated by procedure* A	B	C
0	36–57	44–59	252–297
1	27–32	14–28	225–315
4	46–49	26–37	324–378

* A, Normal swab plating; B, test swab moistened in 1 ml. sterile broth before plating; C, After B, swab was agitated in the remaining broth (0·9 ml.) of which 0·1 ml. was plated in duplicate; the mean number of colonies was then multiplied by 9 to give the total count of organisms released from the swab.

7350 *Bacteroides* organisms (strain GNAB 1) yielded 154, 124, 136, 172, 173 and 152 colony-forming units to 6 successive plates, but on some occasions there was up to a twofold variation in such counts.

The effect of delay in plating the swab

Series of swabs were loaded with the test inoculum in a standard manner and plated on blood agar at various times from 0–4 hr. later. Swabs were held in their plastic tubes on the bench (18–20° C) until plated. At each allotted time, a swab was used to seed 2 well areas on a plate; a second swab was moistened by dipping it in 1 ml. of presteamed sterile nutrient broth in a bijou bottle and the moistened swab was used to seed 2 well areas on a second plate; thereafter, the moistened swab was agitated in the remaining bulk of the 1 ml. of broth in the bijou and two 0·1 ml. volumes of that suspension were plated on replicate plates. Seeded plates were immediately incubated anaerobically. The results of these studies (Table 1) indicate that, with *Cl. welchii*, there was the usual low yield of organisms from a swab (loaded with 1500 bacteria) and this was not improved by moistening the swab before plating. The viable counts estimated from broths into which the swabs were expressed indicate that many organisms remained viable and that there was no obvious progressive inactivation in relation to time spent on the swab.

However, when these studies were extended with some *Bacteroides* strains, it was sometimes possible to demonstrate apparently progressive inactivation related to time spent on the swab. This effect varied with different strains and with the same strain at different times. Replicate swabs were loaded with standard inocula of *Bacteroides* species and plated at 0, 1 and 4–5 hr. In this series, we did not investigate the effect of moistening a swab just before plating, as this is not normal laboratory procedure. Immediately after a standard loaded swab had been used to seed four 'well' areas on plates, it was agitated in 1 ml. of sterile broth; on average, this procedure left 0·9 ml. of a broth suspension of organisms shed ('expressed') from the swab. Two 0·1 ml. volumes of that suspension were spread on replicate plates and the mean number of colonies then obtained was multiplied by 9 to give an 'expressed viability' count (EVC); i.e. the colony count for the 0·9 ml. broth

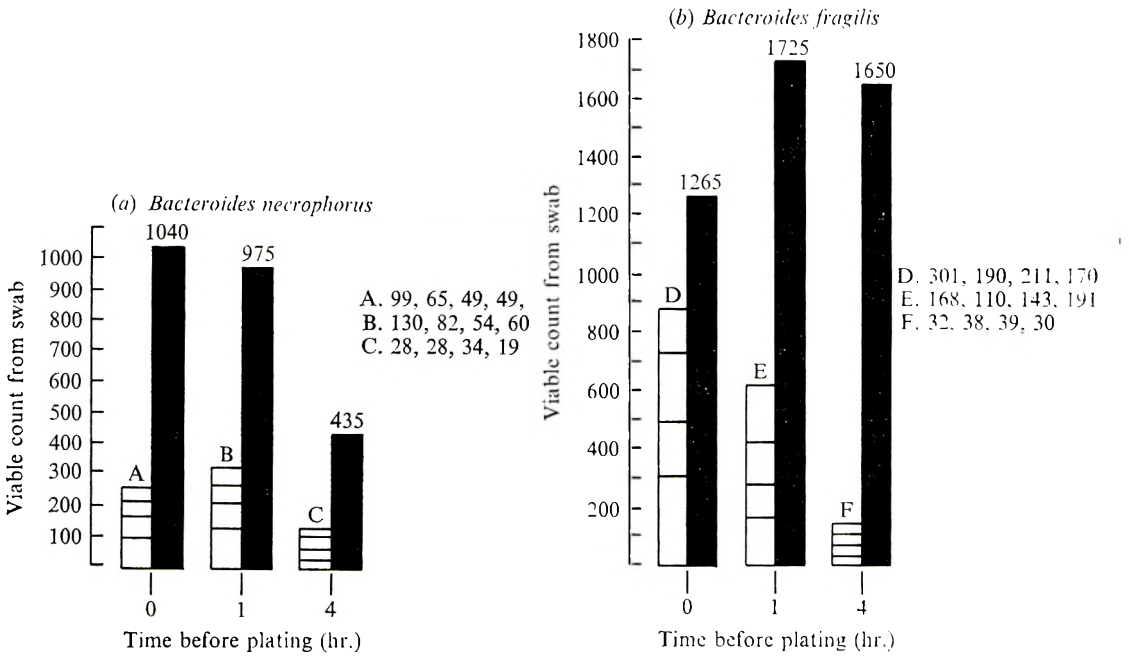

Fig. 1. Comparison of colony counts obtained from a test swab loaded with (*a*) 2860 *Bacteroides necrophorus* organisms, or (*b*) 7000 *Bacteroides fragilis* organisms. The yields from identical swabs plated immediately or held aerobically for 1 hr. or 4 hr. are shown thus □ and the actual counts for each plate are given at A, B, C, D, E, F. Each swab was used at the stated time to prepare four 'well' areas on plates. In each case, swabs were finally expressed into broth and viable counts of the resultant suspensions are shown thus ■ and given at the top of the column.

containing the organisms shed from the swab. The results of the experiments are summarised in Fig. 1. In both of these cases, the plated colony yield had decreased considerably when the delay in plating was 4 hr. The counts obtained from the expressed swab series indicate that *B. fragilis* remained essentially viable whereas the colony counts of *B. necrophorus* were much reduced and in each case the results obtained from plating the swab were much less than the maximum possible yield.

Similar experiments were then repeated by four different operators using replicate swabs each loaded with 6700 organisms of a standard strain of *B. necrophorus*. In general, the results (Table 2) show that replicate platings of a test swab yielded 3–5 % of the load to each well area, but counts varied by up to three-fold in some cases; an occasional high yield of up to 9 % was obtained, and low yields were sometimes less than 1 %.

In all cases, after a loaded swab had been used to prepare 4 well areas, the numbers of viable organisms that could be subsequently expressed from it into fluid (the EVC) indicate that significant proportions of the bacterial loads remained viable and were not released by the normal plating procedure. Thus, with swabs examined after immediate plating, 8–50 % of the original inoculum could be recovered by the expression procedure. With swabs plated after 1 hr., the

Table 2. *The effect of delay in processing on recovery of* B. necrophorus *from test swabs**

Operator no.	Colony counts in four replicate test well areas and the subsequently expressed viability count (EVC) at time (hr.)		
	0	1	4–5
1	280, 283, 323, 230 EVC: **1467**	277, 242, 239, 214 EVC: **1948**	47, 54, 30, 35 EVC: **675**
2	184, 109, 74, 60 EVC: **500**	192, 134, 114, 99 EVC: **1035**	80, 60, 56, 46 EVC: **1048**
3	175, 171, 150, 145 EVC: **1174**	242, 132, 243, 189 EVC: **1570**	166, 147, 87, 92 EVC: **882**
4	280, 195, 178, 181 EVC: **3249**	563, 375, 290, 242 EVC: **2898**	412, 275, 307, 228 EVC: **1278**

* Replicate colony counts and expressed viability counts are derived from swabs each loaded with a simulated exudate containing *ca.* 6700 organisms of *B. necrophorus* and held for periods of 0–5 hr. before processing.

'residual viability' was within that range (16–45 %), and after 4–5 hr. the expressed viability was diminished but was still about 10–20 % in terms of the original inoculum. When this complete series was repeated with the same strain and the same operators, these findings were generally reproducible. Inconsistently good recoveries occurred on two occasions in the expressed series at 4 hr. and one operator was occasionally able to transfer relatively high numbers of organisms to the plate. When the replicate experiments were done with *B. fragilis*, comparable results were obtained and the same sort of variation occurred. Moreover, it became clear that the sustained viability shown by the expressed count in the figure for *B. fragilis* at 4 hr. was not always demonstrable, and that the loss of viability shown for *B. necrophorus* at 4 hr. was not an invariable finding (see the data for operator 2 in Table 2). The variability is clear; the plate count results were obtained with proper attention to the enumeration of adequately large numbers and the expressed viable counts were derived from duplicate plates in each case. The important findings are that the yield obtained from a swab at any time is very poor with this model and that a considerable proportion of the apparent loss is not due to irreversible inactivation.

Non-toxicity of components of the test model

It could be argued that some of the observed inactivation of bacteria on the test swab is attributable to a direct toxic effect of one of the components of the swab or the simulated exudate. Accordingly, identical test inocula of a sensitive *B. necrophorus* strain (NCTC 10575) were exposed for periods of up to 4 hr. at 18–20° C to broth extracts of swab materials, or to broth containing disintegrated swabs, with and without 80 % horse blood. The viable counts obtained were compared with those from control inocula that had been held under similar conditions in nutrient broth. In all cases, there was no demonstrable toxic effect when the test bacteria had been in contact with any of the swab materials, or

extracts of the materials, or horse blood, or with any mixtures of these components. Indeed, the swab materials seemed to have some protective effect for the test bacteria in liquid suspensions.

The effect of aerobic exposure of strict anaerobes on blood agar pending anaerobic incubation

Quantitative studies with *Cl. oedematiens* type D indicate that inactivation of a seeded inoculum on blood agar may proceed quite rapidly (97—98 % loss within 90 min.) if the plates are held aerobically on the bench. For example, a series of standard inocula from an overnight cooked meat broth culture of *Cl. oedematiens* type D were spread on 20 plates of a freshly prepared special solid medium (see Watt, 1973) and immediately transferred to two anaerobic jars that were promptly processed and incubated. A parallel series of plates spread with identical inocula were held aerobically on the bench for 90 min. before being processed anaerobically. The first series gave mean colony counts of $239{\cdot}0 \pm 11{\cdot}1$ and $246{\cdot}6 \pm 9{\cdot}8$. The other series gave a mean count of $5{\cdot}2 \pm 2{\cdot}4$ for one jar and virtually no growth was obtained (2 colonies observed on a total of 10 plates) with the other jar; control plates excluded the possibility that 'jar failure' might account for these differences.

Results of similar studies with *Cl. tetani* and *B. fragilis* show that these organisms are significantly less sensitive than *Cl. oedematiens* under these conditions and there was quite consistent evidence of 'protection on the plate' for these test organisms. For example, replicate blood agar plates spread with essentially vegetative (non-sporing) standard inocula containing *ca.* 250 cells yielded *ca.* 160 colonies of *Cl. tetani* when they were promptly processed; after exposure for 24 hr. before anaerobic incubation, similarly seeded plates subsequently yielded *ca.* 100 colonies. Clearly, vegetative cells of *Cl. tetani* are able to survive aerobic exposure on seeded plates.

Similar studies showed that different *Bacteroides* strains seeded on plates of solid medium varied in their sensitivity to aerobic conditions. With all the test organisms, however, the seeded plates that had been exposed for up to 24 hr. before anaerobic incubation showed reductions of less than 50 % in the colony counts; with some, e.g. *Bacteroides* strains GNAB 7 or 21, there was no reduction in the numbers of colonies obtained on seeded plates held aerobically for 24 hr. before anaerobic incubation.

In studies of total anaerobes in dilutions of human faeces, there was again a considerable degree of survival during aerobic exposure of the seeded plates. In this special case, sterile blood agar plates were held anaerobically for up to 20 hr. so that they were 'reduced' before receiving the inocula. Such a procedure might help to minimize losses attributable to transient aerobic exposure. Despite this precaution in these latter studies without swabs, it is clear that a gradual process of inactivation of wild strains of bacteria taken from a natural environment may start as soon as sampling on plates is begun (Table 3) and there is therefore a case for prompt processing. However, the losses that take place at this stage of the processing are very slight compared with those that we have associated with the use of the swab.

Table 3. *The effect of aerobic exposure of seeded plates bearing standard inocula of diluted faecal suspension on the colony counts obtained after subsequent anaerobic incubation*

Time of exposure of seeded plates (hr.)	Mean colony counts ± S.E.*
0	513·5 ± 36·9
½	503·5 ± 33·6
1	455·8 ± 22·3
2	432·2 ± 19·0
4	387·4 ± 12·1
7	401·8 ± 12·4
24	249·1 ± 12·4

* Each colony count is expressed as the mean ± standard error (S.E.) of the mean derived from 10 replicate plates of prereduced blood agar, each seeded with 0·02 ml. inocula of a ($8·0 \times 10^5$) dilution of human faeces.

DISCUSSION

Many workers have considered possible mechanisms of inactivation of organisms sampled on swabs and most have taken account of potentially toxic primary or secondary factors in the swab material or in the stick (Pollock, 1947, 1948; Rubbo & Benjamin, 1951; Beakley, 1957; Rowatt, 1957; Anderson, 1965; Mair & McSwiggan, 1965; White, 1965; Dadd, Dagnall, Everall & Jones, 1970).

Deleterious influences such as desiccation and overgrowth and other aspects of loss or inactivation in transit have been studied (Cooper, 1957; Crookes & Stuart, 1959; Huet & Bonnefous, 1960; Bartlett & Hughes, 1969; Dadd *et al.* 1970) and various transport media have been developed for delicate organisms (e.g. those of Venkatraman & Ramakrishnan, 1941; Stuart, 1946, 1956; Cary & Blair, 1964). When especially delicate organisms are involved, the culture medium is sometimes seeded directly from the patient and promptly incubated, as is the practice with some cases of gonococcal infection for example. It is surprising that analogous precautions are not routinely associated with the submission of specimens that may contain strictly anaerobic pathogens, particularly when there is a degree of clinical urgency.

Anaerobic infections occur fairly commonly in hospital practice; as laboratory help with these cases is often slow or inadequate, our traditional bacteriological procedures should be critically re-appraised. The laboratory model for the swab studies that we have developed might be challenged on several grounds. Relatively small numbers of organisms from a stationary-phase pure culture were held on a non-toxic swab in a simulated exudate, whereas there might be physiological, immunological and chemotherapeutic factors operating separately or in combination in a 'natural' exudate; moreover there may be bacterial competition within the mixed flora that often occurs naturally. Such important reservations indicate a real requirement for relevant clinical data, though quantitative studies with clinical material along the lines of the present work will be difficult. Meanwhile, several important points emerge from the present study.

The general finding is that, when a simulated exudate containing some thousands of anaerobic organisms was taken up on a swab and plated on solid medium, the yield in terms of the numbers of colonies that we could grow was only about 3–5 % of the sample. This is not an index of toxicity of the swab for the delicate anaerobes that we used. The test swab was non-toxic and the finding was reproducible with an unexacting aerobic organism included in our tests. When a similar test swab was successively plated on separate plates, it continued to release about 3–5 % of its load in terms of colony-forming units per plate for several plates. Thus, the swab appears to be an inefficient sampling device if the aim is to get as many organisms as possible on to the first plate and this may be of importance for the success of a primary culture. In turn, a primary culture is essential if a really prompt diagnosis is to be made in many cases. However, if our aim is merely to get a sample dose on several plates, the swab has some merit – provided that 3–5 % of its load provides acceptable numbers of colonies. It seems that the usual laboratory practice of finally seeding the swab into broth, or breaking it off into the broth, is well based – though this procedure does not allow prompt bacteriological diagnosis and usually leads to the complications that attend the selection of likely pathogens from mixed cultures.

When the loaded swab was agitated in 1 ml. of sterile broth, we could actually express considerably better numbers of organisms from it and we could show that these were viable. This finding is of immediate importance as it illustrated that at least a proportion of the losses in transit that might be attributed to death of delicate organisms is really attributable to non-release of organisms onto plates (i.e. retention on the swab). In addition, our results suggest that some species progressively die on the swab exposed to aerobic conditions. It may be that such 'death in transit' is due primarily to desiccation rather than to aerobic inactivation and we are investigating the various factors involved in such losses. As the swab yielded relatively few colonies to the solid medium in our studies and as its load loses viability in transit, the traditional procedure of plating a swab on a series of primary plates seems to have little chance of prompt success unless large numbers of organisms present in an exudate are plated promptly. There might be a good case for prompt subculture from a very young anaerobic broth culture after about 4–8 hr. incubation, so that the swab's potential is fully exploited before overgrowth with a mixed flora obscures the important evidence.

The fact that some strictly anaerobic bacteria can survive aerobic exposure when seeded on plates pending anaerobic incubation is of considerable interest, but we report this with caution lest it is taken as a general excuse for further delay in the laboratory. Although the results of our current studies (Watt, Collee & Brown, to be published) indicate that ultraspecialized equipment such as an anaerobic cabinet does not seem to be necessary for the laboratory processing of strict anaerobes, we stress the importance of prompt and careful attention; the possible adverse effects of delays in conventional processing are clearly evident in the present work.

It is generally accepted that work with anaerobic bacteria is difficult. Our present evidence suggests that we may start at a disadvantage when we use a

swab; we may greatly add to the disadvantage of accepting a 'normal' delay in transit while the inoculum is not protected on the swab; and we may further increase the disadvantage when we process the swab on primary plates in the traditional manner.

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Hazards involved in the use of furazolidone for the prevention of salmonellosis in broiler chickens

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SUMMARY

The purpose of this work was to study the effects of interrupted, continuous and post-salmonella inoculation treatment with furazolidone in the feed on the colonization of *Salmonella infantis* in the intestines of chickens, as well as the influence of furazolidone *in vitro* on the effect of a mixed culture used for the prevention of salmonellosis in chickens.

It was shown that chickens given interrupted treatment with 0·01 % furazolidone had significantly more salmonellas in the caeca than either chickens fed continuously with this drug or chickens without any treatment. The use of 0·01 % furazolidone after inoculation with *Salmonella infantis* had no effect on *Salmonella infantis* in the caeca of chickens.

The mixed bacterial culture from the normal intestinal flora lost its preventive effect on salmonellosis when cultured with 0·01 % furazolidone.

INTRODUCTION

Broiler chickens reared in the modern way have previously been shown to be highly susceptible to colonization by *Salmonella infantis* (Nurmi & Rantala, 1973). Furazolidone has been used in the feed of broiler chickens as a preventive drug. There is, however, considerable evidence of various hazards connected with the use of antibiotics and chemotherapeutics, one of which is their possible unfavourable effect on the normal intestinal flora. This effect may involve an enhanced susceptibility to certain infections.

Treatment with furazolidone is a fairly easy remedy in cases of manifested infections caused by certain salmonella strains other than *Salmonella infantis*, as has been shown by Wilson (1955) and Perry, Herbert & Braemer (1972). Salmonellas in the intestines of symptom-free carriers are more difficult to eliminate, according to Knivett & Tucker (1972). *Salmonella infantis*, which caused a widespread outbreak of salmonellosis in broilers in Finland in 1971, usually colonizes the intestines of birds without any signs of illness.

The purpose of this work was to study the effect of interrupted treatment with the most commonly used dose of furazolidone (0·01 %) in the feed on the susceptibility of broiler chickens to *Salmonella infantis*, and to test the effect exerted on chickens experimentally inoculated with *Salmonella infantis* by this drug when continuously used, on one hand both before and after inoculation and on the other hand after inoculation only. The purpose of the last part of the work was to study the effect of furazolidone on the growth of the cultured intestinal flora which inhibits the colonization of *Salmonella infantis* in the intestines of chickens. The preventive effect of the intestinal flora cultured from adult cocks has been described earlier (Rantala & Nurmi, 1973).

MATERIAL AND METHODS

The chickens used in the work were the offspring of Cornish cocks and Pilch de Kalb hens. The basal feeds contained no antibiotics or coccidiostats. *Salmonella infantis* was isolated from a field case.

Media used for the detection of salmonellas were bromo-thymol blue–lactose–saccharose–agar, triple-sugar–iron–agar and urea–agar. Selenite broth was used for enrichment. All these were products of the Orion Pharmaceutical Co., Helsinki, Finland. The sera used for typing were produced by Behring-Werke AG, Marburg-Lahn, Germany. The furazolidone came from Pharmacia, Uppsala, Sweden. The feeding of the chickens was started at the age of 2 days in all experiments.

Expt 1. Ten chickens were fed with 0·01 % furazolidone in the feed during 1 week from the age of 2 days onward (group A). Another 10 chickens were kept as controls and fed with the same feed, without furazolidone (group B). The treatment with furazolidone was discontinued at the age of 9 days, and all chickens were inoculated with 10^6 *Salmonella infantis* 1 day later. The chickens were killed 1 week after the inoculation.

Expt 2. Twenty chickens were fed with 0·01 % furazolidone in the feed during the first week after hatching, from the age of 2 days onward. Ten chickens were fed without furazolidone. The furazolidone treatment was interrupted at the age of 1 week in the case of 10 chickens. One day later all chickens were inoculated with 10^3 *Salmonella infantis*. The other 10 chickens from the group fed with furazolidone initially received the same treatment all the time. All chickens were killed at an age *ca.* 2 weeks and studied for salmonellas. There were thus three groups:

Group A: Chickens fed all the time without furazolidone (control group).

Group B: Chickens fed with 0·01 % furazolidone in the feed before inoculation with salmonellas, but not subsequently.

Group C: Chickens fed all the time with 0·01 % furazolidone.

Expt 3. Twenty chickens were inoculated with 10^3 *Salmonella infantis* at the age of 2 days. Feeding was started after the inoculation. Ten of the chickens were then treated during 1 week with 0·01 % furazolidone (Group A), while the other ten received no treatment (group B). All chickens were killed at the age of 9 days and examined for salmonellas.

Expt 4. Six chickens in group A were pretreated at the age of 1 day with a flora cultured from the intestinal contents of adult cocks. The cultivation was performed

Table 1. *Number of salmonellas in the caeca of experimentally infected chickens fed with furazolidone before inoculation, compared with chickens fed without furazolidone. The chickens were inoculated with* 10^6 Salmonella infantis *at the age of* 10 *days and killed* 1 *week later.*

	Number of chickens with different salmonella counts	
	Group A	Group B
No. of salmonellas in 1 g caecal contents	Treated with furazolidone	No treatment
10^8–10^9	1	0
10^7–10^8	9	4
10^6–10^7	0	0
10^5–10^6	0	0
By enrichment only	0	2
Not detected	0	3
Total no. of chickens	10	9

The difference between groups A and B is significant according to the t-test ($t = 2{\cdot}9$, $P < 0{\cdot}01$).

in the VL-medium described by Barnes & Impey (1971), transferring twice to a new medium. The chickens in group B received an identical treatment except that the VL-medium contained 0·01 % furazolidone. Group C served as a control, without pretreatment. All chickens were inoculated with 10^3 *Salmonella infantis* at the age of 2 days and were killed at the age of 1 week.

The caeca of all chickens in each group were studied for salmonellas quantitatively and by enrichment.

The statistical method used was Student's t-test with logarithmic transformation of the values.

RESULTS

In Expt 1 it was shown that chickens fed with furazolidone before inoculation with *Salmonella infantis* had significantly more salmonellas in the caeca 1 week after the inoculation than the controls, which were fed all the time without furazolidone. During the experiment 1 chicken in the control group died. The figures are presented in Table 1.

The chickens in Expt 2 showed the same results as in Expt 1, but with a smaller salmonella inoculum. The chickens fed all the time with furazolidone had eliminated salmonellas as effectively as the controls. The figures from this experiment are given in Table 2.

The third experiment was performed in order to determine the effect of 0·01 % furazolidone used only after inoculation with *Salmonella infantis* on the number of salmonellas in the intestines of chickens. There was no difference between the treated group and the control group. One chicken in the unmedicated group died during the experiment. The counts are given in Table 3.

Expt 4 showed that a culture grown with 0·01 % furazolidone in the medium had no preventive effect on the colonization of salmonellas, whereas the culture

Table 2. *Effect of interrupted feeding of furazolidone on* Salmonella infantis *in the caeca of chickens, compared with continuous treatment with furazolidone, and no treatment. The chickens were inoculated at the age of* 1 *week with* 10^3 Salmonella infantis *and killed* 1 *week later*

	Number of chickens with different salmonella counts		
	Group A	Group B	Group C
No. of salmonellas in 1 g of caecal contents	No treatment	Interrupted treatment with furazolidone	Continuous treatment with furazolidone
10^8–10^9	0	1	0
10^7–10^8	0	3	0
10^6–10^7	1	1	0
10^5–10^6	0	2	0
10^4–10^5	0	2	0
By enrichment only	0	1	0
Not detected	9	0	10
Total no. of chickens	10	10	10

The differences were highly significant according to the t-test between groups A and B ($t = 7{\cdot}1$, $P < 0{\cdot}001$) and between groups B and C ($t = 11{\cdot}0$, $P < 0{\cdot}001$).

Table 3. *Effectiveness of* 1 *week's use of* 0·01 % *furazolidone in the feed on the* Salmonella infantis *after inoculation with* 10^3 *organisms to chickens at the age of* 2 *days*

	Number of chickens with different salmonella counts	
	Group A	Group B
No. of salmonellas in 1 g of caecal contents	0·01 % furazolidone in the feed	No treatment
10^9–10^{10}	0	1
10^8–10^9	10	8
Not detected	0	0
Total no. of chickens	10	9

There is no significant difference between the two groups.

grown without antimicrobial additives was highly effective in this respect. All the chickens pretreated with the culture grown with furazolidone had more than 10^7 salmonellas in 1 g. of caecal contents, as did also the chickens in the control group; no salmonellas were detectable in the caeca of chickens pretreated with the culture grown in the same way as the preceding culture, but without furazolidone.

DISCUSSION

The first two experiments show that if the feeding of a normal furazolidone dose is interrupted, increased susceptibility to salmonellosis may result. The chickens fed without this drug have all the time a resistance against salmonella colonization which is significantly higher than that in the group receiving the interrupted

Table 4. *The effect of pretreatment with flora cultured from intestines of an adult cock both with and without furazolidone on the number of* Salmonella infantis *detected in the caeca of experimentally infected chickens at the age of* 1 *week*

	Number of chickens with different salmonella counts		
	Group A	Group B	Group C
No. of salmonellas in 1 g of caecal contents	Pretreatment with flora cultured without antibiotics	Pretreatment with flora cultured with 0·01 % furazolidone	No pretreatment
10^9–10^{10}	0	2	0
10^8–10^9	0	3	6
10^7–10^8	0	1	0
10^6–10^7	0	0	0
Not detected	6	0	0
Total no. of chickens	6	6	6

The difference between groups A and B was highly significant according to the t-test ($t = 27$, $P < 0{\cdot}001$). There was no significant difference between groups B and C.

treatment. The chickens fed with furazolidone all the time were as resistant as the control groups against the salmonella dose used. The susceptibility of some animals to various infections following therapy with antibiotics is well known. The preventive influence of the normal intestinal flora on the colonization of *Salmonella infantis* in chickens has previously been shown (Nurmi & Rantala, 1973). The results obtained in the experiments described here may thus be due to a disturbance in the development of the normal intestinal flora. This has not been shown to occur with the usual feed additives, such as zinc bacitracin or nitrovin. The results of the experiments with furazolidone show that the use of furazolidone in broiler feeds may result in an increased risk of salmonellosis after the treatment. The treatment should in any case be discontinued before slaughter in view of the possible residues in the meat. The period varies, depending on the legislation in different countries. It follows that a hazard of infection may be present just before slaughter.

The third experiment reported here shows that the usual dose of furazolidone is not effective against *Salmonella infantis* when used after inoculation. Hence, the potential colonization of *Salmonella infantis* induced by the interrupted use of furazolidone cannot be eliminated by a subsequent use of this drug. The opportunity of the *Salmonella* organisms to effect colonization before the treatment may be the reason for the ineffectiveness of the drug.

When furazolidone was used in the medium in which the mixed culture from the intestines of adult cocks was grown, the culture had no preventive effect on the colonization of *Salmonella infantis* in the caeca of chickens. The chickens pretreated with a culture grown in the same way, but without furazolidone, had no salmonellas in the caeca. It thus seems that furazolidone has an unfavourable effect on that part of the intestinal flora which protects the animal against salmonellosis.

Chickens with colonization of their intestines by *Salmonella infantis* show no signs of infection, although the salmonella count in the intestines may be higher than 10^8 organisms in 1 g. of caecal contents. *Salmonella infantis* is able to cause a severe disease in man, and infected chickens thus constitute a serious problem in food hygiene. In view of the results reported here, some drawbacks appear to be associated with the use of furazolidone, which may require practical measures.

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The persistence of mycoplasmas in the urogenital tract of men in the Antarctic

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SUMMARY

A series of meatal swabs, taken from 17 men over a period of 17 months during their tour at an Antarctic base was examined for mycoplasmas. The number of organisms isolated never exceeded 10^4 and not every specimen from each man yielded mycoplasmas. Nevertheless, *Mycoplasma hominis* was isolated from 71 % and T-mycoplasmas from 59 % of the men at some time during their stay. *M. hominis* persisted in the presence of serum IHA antibody titres of 1/64. Three subjects yielded only *M. hominis* and one only T-mycoplasmas.

Six men had already spent a year at the base when the study began and mycoplasmas were still being isolated from some of them at the end of a 31 month period of isolation. The persistence of mycoplasmas in the male genital tract can therefore be independent of sexual contact. Two modes of persistence are suggested; either a few men act as carriers and reinfect the others by contaminating their environment, or as seems more likely, most men have chronic infections.

INTRODUCTION

Mycoplasmas are found in the genital tracts of neonatal infants (Klein, Buckland & Finland, 1969; Foy, Kenny, Levinsohn & Grayston, 1970), but isolation during childhood is rare. Subsequently, it is possible to isolate these organisms from a proportion of adolescent persons at, and following, puberty (Mårdh & Weström, 1970). In addition, the proportion of children with serum antibodies to mycoplasmas begins to increase at about the time of puberty (Purcell, Chanock & Taylor-Robinson, 1969). In adult life, T-mycoplasmas have been isolated from the genital tract of about 50 % of apparently healthy men and from about the same proportion of non-pregnant women (Taylor-Robinson & Furr, 1973), while *Mycoplasma hominis* has been isolated somewhat less frequently. Isolation of mycoplasmas at puberty suggests that changes in the genital tract and possibly sexual activity are conducive to the establishment of infections. The hypothesis that their subsequent spread is largely the result of sexual activity is reinforced by the findings of Archer (1968), and Kundsin, Parreno & Kirsch (1973), who succeeded in isolating mycoplasmas from the urogenital tracts of only 8 % and 3 % respectively, of two groups of nuns. However, it is unknown whether mycoplasma infections of the male genital

tract persist or whether they are transient. In the latter case, reinfections from repeated sexual encounters could simulate persistence. In an attempt to resolve this problem a group of men were studied during their stay at a British Antarctic Survey base.

MATERIALS AND METHODS

Subjects

The group studied was the wintering party of 1971–72 at Stonington Island, a base on the Antarctic Peninsula in Marguerite Bay. There were 17 men, six of whom had completed 1 year of a 2-year tour. The remaining 11 arrived during the relief of 1970–71 to stay for one or two years. The last ports of call of the ships where social contacts could be made were Montevideo, and Port Stanley in the Falkland Islands. None of the incoming group had any overt venereal disease after leaving these ports. They were studied for a total period of 17 months at the base and on board the Survey's ships.

Specimens and storage

Meatal swabs were collected at 6- to 10-week intervals from January 1971 to May 1972. Each man took his own swabs before micturition. The final samples were taken aboard ship from the six men returning to England. Each swab, of plain cotton wool (Stayne labs), was snapped off in 1·5 ml. of mycoplasma medium (Manchee & Taylor-Robinson, 1968), which contained glucose. On base these specimens were stored at $-28°$ C., during the voyage home at $-40°$ C., and in England at $-70°$ C. Serial blood samples were also taken during the study and the sera shared the same storage conditions.

Mycoplasma isolation

Medium expressed from the swabs was diluted 10^{-1}, 10^{-2}, and 10^{-3} in duplicate portions of three liquid mycoplasma media which contained phenol red and 0·1 % of glucose, arginine and urea, respectively. Detection of mycoplasmas was based on the occurrence of a change in colour of the media (Taylor-Robinson & Purcell, 1966). Medium containing glucose was included not only for the detection of *M. fermentans* but also for *M. canis* and other canine mycoplasmas. In addition, each sample was inoculated onto agar media containing, respectively, arginine and urea. All cultures were incubated at 37° C. for up to 14 days and specimens containing organisms at the 10^{-3} dilution were retested, being diluted up to 10^{-6}.

Where a series of specimens from one subject yielded arginine-metabolizing organisms, a single isolate was selected for identification by the disk inhibition technique (Clyde, 1964) using *M. hominis* (PG21) antiserum.

Serological tests

M. hominis serum antibody titres were determined by the indirect haemagglutination (IHA) micro-technique (Taylor-Robinson *et al.* 1965). Each of the serial sera taken during the year was inactivated at 56° C. for 30 min. and adsorbed with unsensitized tanned sheep red cells at 37° C. for 2 hr. The sera were then titrated at an initial dilution of 1/8 against sensitized tanned cells. Since many of the sera

Table 1. *Persistence of* M. hominis *and T-mycoplasmas in the urogenital tracts of* 17 *men wintering at Stonington Island*, 1971

	Mycoplasmas isolated for at least (months)	Number of men yielding *M. hominis*		T-mycoplasmas	
1st	0	5		7	
year	6	0		1	
men	11	2		3	
	13	0		2	
	16	5		0	
	17	2	71 %	0	59 %
2nd	23	0		2	
year	24	1		0	
men	29	2		1	
	31	0		1	
	Totals	12		10	

in the lower dilutions agglutinated unsensitized tanned cells, controls consisted of duplicate dilutions of the sera tested against them. The sensitized and unsensitized tanned cells had been used successfully in a previous study (Taylor-Robinson *et al.* 1965) and had been stored at $-70°$ C. for the past 9 years. Titres of serum antibody against a T-mycoplasma were determined by the metabolism-inhibition method (Purcell, Taylor-Robinson, Wong & Chanock, 1966). Strain T-27 was used because it had been found previously that 82 % of 50 adult sera inhibited its metabolism to some degree (L. Q. Sang & D. Taylor-Robinson, unpublished observations). The sera were used at an initial dilution of 1/2.

RESULTS

Isolation of mycoplasmas

M. hominis

This mycoplasma was found on one or more occasion during the isolation period in 12 (71 %) of the 17 men (Table 1). However, mycoplasmas were not isolated from every specimen taken from the men harbouring these organisms. Of 72 swab specimens examined, only 23 (32 %) yielded mycoplasmas, and these were present in low numbers since they were not isolated from specimens diluted more than 10^{-3} (Table 2). In nine cases mycoplasmas were isolated from only the lowest dilution of one of the duplicate cultures and of these they were re-isolated from only five of the original specimens, probably due to loss of organisms during freezing and thawing. In only one case, that of subject no. 3, were mycoplasmas isolated from every specimen (Table 2).

The number of positive cultures from each series of samples increased towards the end of the isolation period (Fig. 1). Thus, whereas *M. hominis* was recovered from only three of 15 men in March–April 1971, it was recovered from eight of 17 men sampled in February 1972.

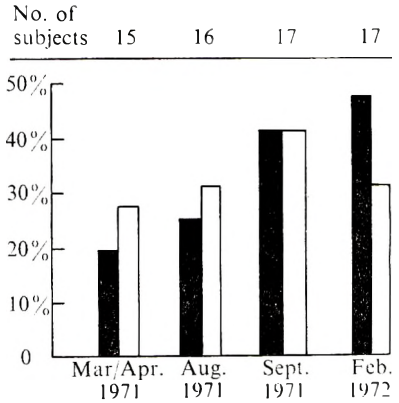

Fig. 1. Percentages of subjects from whom mycoplasmas were isolated at Stonington Island, March 1971–February 1972. ■, *M. hominis*; □, T-mycoplasmas.

T-mycoplasmas

These were recovered on one or more occasions from 10 (59 %) of the 17 men (Table 1). However, of 72 swab specimens examined, T-mycoplasmas were isolated from only 24 (33 %) of them, a result similar to that for *M. hominis*. The number of organisms on each swab was also similar, since they were cultured from only one specimen at a dilution of 10^{-4} (Table 2). Six of these 'positive' specimens yielded mycoplasmas at the lowest dilution in only one of the duplicate cultures. Subject no. 3 (Table 2) was again the only person whose swabs yielded mycoplasmas on every occasion. The number of isolations made from a series of swabs seemed to be unrelated to the particular time of the year during which they were taken (Fig. 1).

Comparison of M. hominis *and T-mycoplasma isolation*

The number of swabs which were positive for *M. hominis* (23) was similar to that for T-mycoplasmas (24) (Table 2). In addition, the numbers of organisms of the two mycoplasma species that were isolated from each swab were similar. However, both mycoplasma species were not always isolated from each swab. Thus, *M. hominis* but no T-mycoplasmas were isolated from subjects nos. 1, 6 and 17, and T-mycoplasmas but not *M. hominis* from subject no. 7. The remaining men from whom mycoplasmas were isolated carried both species at some time during the isolation period.

Antibody to mycoplasmas

M. hominis

The titres of antibody measured by IHA ranged from < 8 to 512 (Table 3). There were fourfold or greater rises in the titres of antibody in the sera of subjects nos. 2, 8 and 13, and one eightfold fall in the sera of subject no. 17. Mycoplasmas were isolated from subjects nos. 2, 13 and 17 but not from subject no. 8. The other 13 subjects had antibody titres of < 8 to 64 and these were maintained without significant change over the period of isolation. *M. hominis* was cultured from nine of these men on one or more occasion; from subjects nos. 1, 3 and 9 at times when their serum antibody titres were 1 in 64, but not from subject no. 5 who had antibody titres of 256–512. There would, therefore, appear to be no correlation between changes in, or the presence of, serum antibody and the isolation of *M. hominis*.

Table 2. *Numbers of organisms isolated, March* 1971 *to April* 1972

		M. hominis						T-mycoplasmas					
	Subject no.	Mar. 71	Apr. 71	Aug. 71	Sept. 71	Feb. 72	Apr. 72	Mar. 71	Apr. 71	Aug. 71	Sept. 71	Feb. 72	Apr. 72
1st year men	1			–	10^1	–	–			–	–	–	–
	3	10^1		10^1	10^2	10^2	10^3	10^2		10^1	10^3	10^2	10^2
	8		–	–	–	–	–	–	–	–	–	–	10^2
	9			–	–	10^3				–	10^1	10^1	
	11	–		10^3	10^1	–		–		10^3	10^3	–	
	13		–	–	–	10^1			–	–	–	–	
	14		10^3	–	10^1	10^2			10^3	–	10^3	10^4	
	15		–	–	–	10^1			10^1	–	10^1	10^1	
	17		–	–	–	10^1			–	–	–	–	
2nd year men	2	10^1	–	–	–	–	–	–	–	–	10^1	10^1	–
	4	–		10^2	10^1	10^1		–		10^2	–	–	
	6		–	–	10^1	10^1			–	–	–	–	
	7	–		–	–	–	–	10^2		10^1	–	–	–
	10	–		10^3	10^1	–		–		10^3	10^3	–	

– = less than 10 organisms/ml. Blank = not swabbed.

Table 3. *Titres of serum antibody to* M. hominis *and T-mycoplasma strain* 27, *March* 1971 *to February* 1972

Subject no.	Antibody to *M. hominis* measured by IHA							Antibody to T-27 measured by MI						
	Mar. 71	Apr. 71	Aug. 71	Sept. 71	Dec. 71	Feb. 72	Rise or Fall	Mar. 71	Apr. 71	Aug. 71	Sept. 71	Dec. 71	Feb. 72	Rise or Fall
1	–	–	32	64	64	64		–	–	< 2	< 2	< 2	< 2	
2	16	32	32	32	64	64	↑	< 2	< 2	8	<	< 2	< 2	↑ ↓
3	64	–	64	32	32	32		< 2/2*	–	4	< 2	< 2	4	↓ ↑
4	64	64	32	32	32	32		< 2	4	4	< 2	< 2	4	↑ ↓ ↑
5	–	256	–	256	512	256		–	8	–	2	4	2	↓
6	–	< 8	< 8	< 8	< 8	< 8		–	2	4	< 2	< 2	< 2	↓
7	< 8	–	8	8	8	8		< 2	–	< 2	< 2	< 2	< 2	
8	–	16	16	32	64	128	↑	–	2/4	4/2	< 2	2/< 2	4	
9	–	32	32	32	64	64		–	2/< 2	< 2	< 2	< 2	2	
10	16	–	32	32	32	32		< 2	–	< 2	< 2	2	2	
11	32	–	32	32	32	32		< 2	–	< 2	< 2	< 2	2	
12	64	–	64	32	32	32		< 2/2	–	2	4/2	< 2/2	< 2	
13	–	< 8	< 8	8	8	16	↑	–	4	2/4	2	4	2	
14	–	16	16	16	32	16		–	< 2	< 2	< 2	2	< 2	
15	–	< 8	< 8	8	8	8		–	< 2	< 2	< 2	2	< 2	
16	–	8	8	8	8	8		–	4/8	2	2	8	8/4	↑
17	–	64	32	32	8	8	↓	–	2	4	< 2	< 2	< 2	↓

– = Serum not available. * Results of duplicate tests.

T-mycoplasmas

Antibody titres to strain T-27 measured by metabolism inhibition were low, ranging from < 2 to 8. There were 5 fourfold or greater rises and six falls in the titre of antibody (Table 3). T-mycoplasmas were isolated from three of the four men who had rises in serum antibody titre, and from three of the seven subjects who had falls in titre. There was no correlation between the changes in antibody titre and the isolation of T-mycoplasmas.

Studies on dogs

Mycoplasmas were not isolated from pharyngeal and genital swabs taken on three occasions over a period of 6 months from 12 of the 140 Husky dogs at Stonington Island. A single set of sera from the same animals did not specifically inhibit the metabolism of *M. hominis*, indicating the absence of antibody. The sera were not tested for antibody to T-mycoplasmas, nor by IHA against *M. hominis*.

DISCUSSION

The average rate of isolation of mycoplasmas from the men, based on swabs taken on any single occasion, is similar to the rate of isolation reported by other workers who studied larger numbers of men without disease (Taylor-Robinson, Addey, Hare & Dunlop, 1969; Taylor-Robinson & Furr, 1973). However, although repeated isolations were made from a series of swabs taken from each individual, these isolations were often intermittent. This was not due to variation in medium because the same batch was used throughout. Failure to isolate mycoplasmas consistently might have been due to a series of transient infections of the genital tract. Each reinfection might have originated in one of three ways. The first, from handling Husky dogs infected by T-mycoplasmas, is unlikely since the available evidence suggests that these dogs did not carry T-mycoplasmas, nor was anything known about the feasibility of dog to man transmission. A second possibility, auto-reinfection of the genital tract by mycoplasmas infecting the oropharynx, is unlikely because only about 5% of persons have the same mycoplasmas in their throats as in their genital tracts (Ford, 1967). Another alternative explanation is the persistence of mycoplasmas in the environment due to the primitive facilities for personal hygiene. Despite the possibility of consecutive reinfections, it is true to say that mycoplasma infections tend to be chronic rather than acute. *M. hominis* is well known to cause chronic contamination of tissue cultures (Stanbridge, 1971). *M. pneumoniae* may persist in the respiratory tract even after the institution of apparently adequate antibiotic therapy (Smith, Chanock, Friedewald & Alford, 1967), and T-mycoplasmas persist for many months in the genital tracts of isolated dogs (D. Taylor-Robinson & P. M. Furr, unpublished observations). It is not unlikely that the same situation exists in the genital tract of man. If this is so, inability to isolate mycoplasmas consistently from the swabs might have been due to inconsistent swabbing, or to the presence of mycoplasmas in small numbers only, so that those on some swabs might not have survived the variable storage conditions ($-28°$ C. for several months before lower temperatures).

It was to be expected that organisms on swabs taken in the early phase of the study would not survive as well as those taken later; in this respect it seems that T-mycoplasmas survived better than *M. hominis*. A third explanation might be that some of the men were given antibiotics for short periods only. These included tetracyclines but, unfortunately, no records of administration are available. Tetracyclines are effective in inhibiting the growth of mycoplasmas; suboptimal doses suppress multiplication rather than eradicate the organisms (Shepard, 1972).

It was hoped that the assessment of antibody titres would help to determine whether or not re-infections had occurred. However, since there was no evident correlation between the presence of, or changes in, serum antibody titres and the isolation of *M. hominis* or T-mycoplasmas, there was nothing to indicate whether or not the intermittent isolations were due to re-infections. The small variations in the titres of antibody to the T-mycoplasmas are difficult to interpret. Apparent rises and falls in titre could be due to the presence of antibiotics in the subjects' sera, or responses to serial infections by different strains.

Most antibody titres persisted unchanged. This would be expected if persistent organisms provided a continuous antigenic stimulus for antibody production. Although the titres of IHA antibody recorded in the present study were similar to those found to mitigate symptoms in volunteers following intranasal inoculation of *M. hominis* (Mufson *et al.* 1965), they are clearly insufficient to eradicate organisms established in the genital tract. It is, therefore, likely that local antibodies and cell mediated immunity are the most important protective mechanisms controlling urogenital mycoplasma infections.

It is likely that T-mycoplasma infections can occur simultaneously with more than one serologically distinct strain, some persisting and others transient. Howard, Gourlay & Brownlie (1973) have observed variable persistence in experiments with bovine strains in the bovine udder. Whether mycoplasma infections of the human urogenital tract, once established, persist for life, obviously cannot be assessed from the present study. It is quite clear, however, that both *M. hominis* and T-mycoplasmas can persist in the male genital tract for at least two and a half years independent of sexual contact.

The authors are indebted to the members of the British Antarctic Survey for their co-operation in this study, to the survey itself for providing equipment and facilities, and to Mr G. Thompson for valuable technical assistance.

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Corynebacterium pyogenes and bovine abortion

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SUMMARY

In the examination of bovine fetal material it was found that there was a significant increase in the proportion of mixed infections identified as the time between abortion and the collection of the samples increased. Examination of paired serum samples from abortions from which only *Corynebacterium pyogenes* was isolated revealed evidence of active infection in two-thirds, suggesting that *C. pyogenes* may have been acting as a primary abortifacient in these cases.

INTRODUCTION

The association of *Corynebacterium pyogenes* infection with bovine abortion is well known. The literature has been reviewed (Hinton, 1972). Abortion has been produced experimentally in both cattle (Reisinger, 1928; Johnson & Graham, 1945; Kolar, 1963) and sheep (Sørum, 1953; Smith, Reynolds, Clark & Milbury, 1971). The role of *C. pyogenes* as an abortifacient is not clearly understood though a possible pathogenesis has been suggested (Hinton, 1972).

Kolar (1963) isolated *C. pyogenes* from the fetus in 204 cases of abortion and he noted that the prognosis in these cases was often poor. Retention of the fetal membrane, either with or without purulent metritis occurred in 60 % and 45 % of cases respectively and many of these cows were subsequently sterile. A quarter of cows developed either fever, anorexia, metritis, pneumonia, mastitis or arthritis after abortion and were sent for emergency slaughter. In only a fifth of cases were there no apparent secondary complications.

Lovell (1939) found that though the serum of apparently normal cattle contained low titres of antibodies to *C. pyogenes* haemolysin (toxin) the titres were frequently raised in cases of mastitis or suppuration associated with *C. pyogenes* infection and were also raised in the only abortion examined. On the other hand in post-parturient endometritis, a condition in which *C. pyogenes* is usually considered to be a localized secondary and ascending infection, the antihaemolysin titres are rarely raised much above Lovell's normal titres (Dawson, 1951).

In view of Kolar's disturbing report it would seem that abortion associated with *C. pyogenes* infection merits further study. This paper gives the circumstances in which *C. pyogenes* was isolated from 100 such cases and also gives the results of examining sera using a serum antihaemolysin test.

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MATERIALS AND METHODS

Abortion cases

The 100 cases from which *C. pyogenes* was isolated were identified during the routine examination of bovine abortion material at the Carmarthen Veterinary Investigation Centre. *C. pyogenes* was isolated from either fetal membrane (placenta), fetal stomach or swab of vaginal mucus. The strains of *C. pyogenes* were identified on the basis of their colonial and morphological characteristics and their ability to produce beta-haemolysis and to liquify solid serum.

Paired serum samples were examined from 21 of these cases for antibodies to *C. pyogenes* haemolysin. In addition paired sera from 20 abortions which showed no evidence of *C. pyogenes* infection were screened by way of controls.

Haemolysin production

Haemolysin was produced by growing a subculture of *C. pyogenes* strain NCTC 5224 in litmus milk (Oxoid Ltd.) for 48 hr. at 37° C. The haemolysin (toxin) in the whey was titrated against 2 % rabbit erythrocytes (r.b.c.) and the volume adjusted to 50 MHD/ml. with CFT dilutent (Oxoid Ltd).

Serum haemolysin neutralization test

The serum was diluted in 0·5 ml. of the haemolysin solution and incubated for 1 hr. at 37° C.; 0·5 ml. 2 % rabbit r.b.c. in CFT dilutent were added and after a further 2 hr. incubation the wells were examined for haemolysis by placing the perspex trays over a diffuse light-source. The final dilutions ranged between 1/10 and 1/20480.

Both samples of a pair were tested together using the same batch of reagents. The serum antihaemolysin titre was taken as the reciprocal of that dilution of serum which when incubated with 25 MHD of haemolysin for 1 hr. prevented 50 % haemolysis. A significant change in titre has been taken to be at least fourfold either up or down.

RESULTS

The isolation of C. pyogenes *from bovine abortions*

The results obtained from the 100 cases is listed in Table 1. These show that as the time between the abortion and the collection of the specimens increases so does the chance that a mixed infection will be identified. This difference is significant ($P < 0{\cdot}001$).

The serum haemolysin neutralization test

The results of the serum haemolysin neutralization test in the 21 cases of abortion associated with *C. pyogenes* infection are listed in Table 2.

The cases can be divided into four groups on the basis of the serological response. In Group I (10 cases) there was a fourfold or more rise in serum antihaemolysin titre while in the three cases in Group II the titres fell by at least eightfold.

In the two cases in Group III there were high titres at both samplings while in

Table 1. *The isolation of* Corynebacterium pyogenes *and other pathogens from* 100 *bovine abortions which yielded* C. pyogenes *on culture*

Days after abortion*	No. of cases	Material cultured (No. positive for *C. pyogenes*/No. examined)			Cases with mixed infection			
		Fetus	Fetal membrane	Vaginal mucus	*Br. abortus*	Mycotic	*S. dublin*	Total (%)
1	31	8/8	24/24	7/9	0	1	0	1 (3)
2	25	1/3	24/24	3/3	2**	4	0	6 (24)
3–7	35	1/1	26/26	11/12	7	3	2	12 (34)
8–20	9	—	3/3	6/6	0	2	1	3 (33)
Total	100	10/12	77/77	27/30	9	10	3	22 (22)

* Day 1 = first 24 hr. after abortion.
** In one of these cases a mycotic infection was also demonstrated.

Table 2. *The antihaemolysin titre in* 21 *cases of bovine abortion associated with* Corynebacterium pyogenes *infection*

Group	Sample yielding *C. pyogenes* on culture at first examination*			Day of sampling**		Antihaemolysin titre		Change in titre		Other pathogens isolated
	FM	FS	VM	1st	2nd	1st	2nd			
I	+			1	25	40	320	+	8	
	+			1	14	80	320	+	4	
			+	1	14	160	640	+	4	
	+	+		1	20	1280	5120	+	4	
	+	+		1	23	1280	10240	+	8	
	+			2	15	10	80	+	8	
	+	−		2	21	160	5120	+	32	
	+			3	17	40	5120	+	128	
	+			3	19	640	2560	+	4	
	+		−	5	17	160	1280	+	8	
II	+			1	15	2560	40	−	64	
	+	+		4	17	10240	320	−	32	
	+			7	15	1280	160	−	8	*S. dublin*
III	+			5	19	5120	2560	−	2	
	+			5	20	1280	640	−	2	
IV	+			2	11	40	40		0	
	+			2	14	80	80		0	*A. fumigatus*
	+			4	17	10	10		0	*A. fumigatus*
			+	4	15	40	40		0	
			+	4	12	80	80		0	
	+			6	14	80	80		0	

* FM = fetal membrane. FS = fetal stomach. VM = vaginal mucus.
** Day 1 = first 24 hr. after abortion.

the six cases in Group IV there was no evidence of active infection with the titres remaining unchanged at $\leqslant 80$.

In 18 of the 20 control cases the titres were $\leqslant 80$ at both samplings and these showed no significant change. One of the other cases was classified in Group I

because the titre rose from 40 to 160 while the last had titre of 640 at both samplings and was classed as Group III. The difference in the distribution of 'infected' and 'control' cases in the four groups is highly significant ($P < 0{\cdot}001$).

DISCUSSION

The examination of paired serum samples revealed that in two-thirds of cases in which *C. pyogenes* was the only significant bacterial or fungal isolate there was a significant change in anti-haemolysin titre. This indicates that the abortion and the infection were probably specifically associated and consequently *C. pyogenes* could have been acting as a primary abortifacient. However, it is appreciated that the interpretation of this serological test must be approached with caution because *C. pyogenes* is a common pyogenic organism of cattle and that the specificity of the test has not been fully assessed.

In addition, there was evidence that *C. pyogenes* may also act as a secondary invader in that the proportion of mixed infections increased significantly as the time between the abortion and the collection of the specimens increased, and because *C. pyogenes* may be isolated without serological evidence of infection, as occurred in four cases in Group IV.

Obviously further work needs to be done on this condition so that reliable diagnostic criteria can be established and the implications of Kolar's (1963) observations can be evaluated on a much wider scale.

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The growth of salmonellas on cooked cured pork

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SUMMARY

With the use of streptomycin-resistant mutants to facilitate recovery, 5 strains of 4 species of *Salmonella* were shown to grow rapidly at 22° C. on low salt ham even from an inoculum of 10–20 organisms. *S. pullorum* did not grow well. All 6 strains of *Salmonella* survived but did not grow on 'high salt ham'. We conclude that cooked ham containing approximately 2·8 g. NaCl/100 g. H_2O once infected is more likely to give rise to food poisoning than is ham with the higher salt content traditionally used.

INTRODUCTION

Large doses of salmonellas are required to initiate food poisoning; doses that can arise only through massive contamination or through light contamination followed by the opportunity for growth before ingestion. Foods responsible for salmonella food poisoning are frequently not identified (e.g. the source of salmonellas was detected in only 20 % of the recorded general outbreaks and 2 % of the recorded family outbreaks in 1966 in England and Wales; Vernon, 1967) and so general vigilance is required. Rare outbreaks associated with cooked cured pork have been reported (Wildman, Nicol & Tee, 1951; Bailey *et al.* 1972), but the possibility of more ready growth of salmonellas on some such products arising from the recent tendency to use less sodium chloride than traditionally used appears not to have been adequately assessed. This paper reports a study of the growth of salmonellas on such a product and on a more traditional cured pork and assesses the effect of various factors. Previous studies have involved adding salmonellas to 1 % glucose in sterile jelly from ham (Koelensmid & van Rhee, 1964) or to ground pork containing the normal flora (Alford & Palumbo, 1969). However, in our opinion the ability of salmonellas to grow on meats should be assessed not by using artificial preparations but by using the meat in slices in the form in which it would be purchased, and the inoculum should be labelled in some way so that it is not confused with any salmonellas already on the meat. Therefore, to determine the ease with which salmonellas could grow on two different types of cooked cured pork we used streptomycin-resistant mutants inoculated on portions of the meat as purchased from shops.

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METHODS

Organisms

Salmonella enteritidis 4753/70, *S. typhimurium* 4718/70 and *S. newport* 3313/70, all isolated from raw meat and kindly provided by the Salmonella Reference Laboratory, Colindale Avenue, London, N.W.9 and *S. enteritidis* NCTC5765, *S. choleraesuis* NCTC5736 and *S. pullorum* NCTC5776 were used. Slide tests showed that each strain was agglutinated by the appropriate group specific O antiserum (Wellcome Reagents, Beckenham, Kent). Other organisms were *Escherichia coli* NCTC8984, 8986, 9434, 10487, 10537, all of which were stated to grow well at 22° C., *E. coli* 16A (from our departmental collection) and *Proteus vulgaris* NCTC10015.

Media

Figures refer to % (w/v) in demineralized water. Nutrient broth was peptone (Evans), 0·5; Lab Lemco (Oxoid), 0·5; NaCl, 0·5; sterilized at 121° C. for 20 min.; pH 7·0. Nutrient agar was No. 3 agar (Oxoid), 1·2; other ingredients as for nutrient broth; sterilized at 121° C. for 20 min.; pH 7·0. MacConkey agar was CM7 (Oxoid). Glucose yeast Lemco agar (GYLA) was peptone (Evans), 1·0; Lab Lemco (Oxoid), 1·0; yeast extract (Difco), 0·25; Japanese shredded agar, 2·0; glucose, 0·5; sterilized at 121° C. for 15 min., pH 7·0. Minimal agar was minimal agar of Clowes & Hayes (1968) with 0·2% lactose substituted for glucose. Ringer's solution ($\frac{1}{4}$ strength) was prepared by dissolving 1 BR 52 tablet (Oxoid) in 500 ml. glass distilled water and sterilizing at 121° C. for 15 min. Streptomycin sulphate B.P. (Glaxo, Greenford) was used, concentrations being expressed in terms of amount of streptomycin base.

Production of streptomycin-resistant mutants

Streptomycin-resistant mutants (str^r) were produced by the method of Meynell & Meynell (1970), by adding 100 ml. nutrient broth containing 2000 μg./ml. streptomycin to an overnight culture at 37° C. of each of the parental streptomycin-sensitive salmonellas (str^s) in 100 ml. nutrient broth and plating samples from the cultures after further incubation for 8 hr. and 24 hr. on nutrient agar containing 1000 μg./ml. streptomycin. After incubation of the plates for 24 hr. a single colony of each strain was used to establish stock cultures of str^r mutants, identity being confirmed by slide agglutination. Maintenance was on nutrient agar slopes from which streptomycin was omitted to stop development of streptomycin dependance, periodic counts on several media indicating that reversion to str^s was not a problem.

Cured meat samples for growth experiments

Over 6 months, samples of prepacked sliced cooked shoulder of cured pork were obtained in $3\frac{3}{4}$ oz. packs containing 4 slices from a refrigerated display cabinet at a supermarket. The manufacturing specification for this product was: NaCl, 1·8–2·2% (w/w); KNO_2, 60 p.p.m.; no nitrate; total colony count less than 500/g. This material had a water content of *ca.* 70% and was called by us 'low salt

ham'; analysis for Cl^- using an automated application of the mercuric thiocyanate and ferric alum reagents confirmed that the NaCl content was *ca.* 2 % (w/w) i.e. *ca.* 2·8 g./100 g. H_2O. For comparison samples of cooked shoulder of cured pork sliced and packed at the point of sale were obtained from a small store. This product contained *ca.* 4 % (w/w) NaCl i.e. *ca.* 6 g. NaCl/100 g. H_2O and was called by us 'high salt ham'.

Inoculation and incubation of meat

Overnight broth cultures at 37° C. in a shaking water bath contained *ca.* 1×10^9 salmonellas/ml. Cultures were diluted in aqueous NaCl (0·5 %), either alone or as mixtures, to give approximately the required number of organisms in 0·02 ml. for inoculation. Meat free from fat was aseptically cut with a No. 13 cork borer into pieces 20 mm. diam. which weighed about 0·5 g. and were about 2 mm. thick, and placed in Petri dishes. Pieces were inoculated with 1 drop (0·02 ml.) of cell suspension from a calibrated pipette. Petri dishes containing the portions were then incubated in polythene bags aerobically at 5, 10, 17 or 22° C. The number of organisms in the inoculum was determined by making colony counts on the suspension and on one of the pieces immediately after inoculation.

Counts of viable bacteria

A piece of meat was transferred to a 1 oz. universal bottle which contained 10 ml. Ringer's solution and blended at *ca.* 14,000 rev./min. for 3 min. using a top drive homogenizer (M.S.E., London S.W.1). Appropriate dilutions (0·1 ml.) of the supernatant fluid in Ringer's solution were inoculated on plates (2/dilution) that had been dried at 55° C. for 40 min. and spread. Counts of bacteria in liquid media were performed similarly. For counts of salmonellas, nutrient agar + 1000 μg. streptomycin/ml. incubated overnight at 37° C. was used; for *E. coli*, minimal agar incubated at 37° C. for 48 hr. was used; for *P. vulgaris* MacConkey agar incubated overnight at 37° C. was used – no swarming occurred; and for general colony count, GYLA incubated at 22° C. for 3 days was used. Counts were expressed as the number of organisms per piece of meat or per ml. of liquid medium.

Growth of organisms in liquid media

Nutrient broth (15 ml. in conical flask with attached test tube for nephelometry, or 50 ml. in 250 ml. conical flasks) with various additions was inoculated with 0·5 or 1·0 ml. of overnight shaken culture at 37° C. and was incubated at 22° C. Growth was estimated by colony counts or by relative turbidity using a nephelometer with filter OR2 (Evans Electroselenium Ltd., Halstead, Essex).

RESULTS

Suitability of streptomycin-resistant mutants for studying growth rates of salmonellas on cured meats

Comparisons by nephelometry of growth rates in shaken nutrient broth of str^r mutants and str^s parental strains of salmonellas indicated that there was little difference between the two, whether the broth was incubated at 22 or 37° C. or

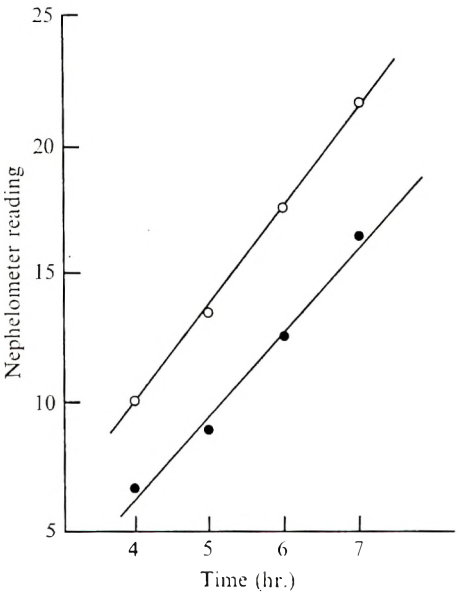

Fig. 1

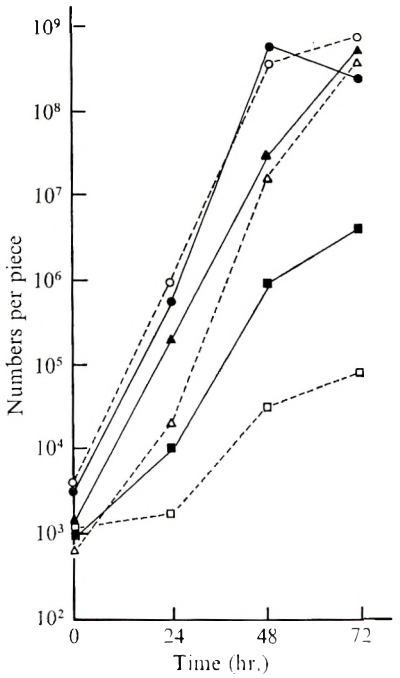

Fig. 2

Fig. 1. Comparative growth rates at 22° C. of *Salmonella enteritidis* NCTC 5765 str^s wild type and an str^r mutant in nutrient broth containing 3 % (w/v) NaCl. Each point is the average of nephelometer readings given by 2 flasks. ○, *S. enteritidis* str^s; ●, *S. enteritidis* str^r.

Fig. 2. Growth of str^r mutants of various salmonellas on 'low salt ham' at 22° C. ●, *S. enteritidis* 4753/70; △, *S. enteritidis* NCTC5765; ○, *S. newport* 3314/70; ▲, *S. typhimurium* 4718/70; ■, *S. choleraesuis* NCTC5736; □, *S. pullorum* NCTC5776.

contained 0·5 % or 3 % NaCl (e.g. Fig. 1). No evidence was obtained of reversion of str^r to str^s and so we concluded that str^r mutants could be used to study the growth rate of salmonellas on cured meats.

Survival and growth of salmonellas on cured meat

When inoculated on to separate pieces of 'low salt ham' incubated at 22° C. numbers of *Salmonella enteritidis* NCTC5765, *S. enteritidis* 4753/70, *S. choleraesuis* NCTC5736, *S. newport* 3314/70 and *S. typhimurium* 4718/70 increased from 10- to more than 100-fold within 24 hr. The increases continued with further incubation. *S. pullorum* showed virtually no growth at 24 hr. and only slight growth thereafter. Typical results are shown in Fig. 2. Experiments with *S. enteriditis* NCTC5765 indicated that the initial rate of growth was similar, irrespective of the number of organisms in the inoculum (Fig. 3), though with a smaller inoculum the number of salmonellas after 3 days was less than with a larger inoculum, presumably because other organisms already on the ham were able to grow to constitute a larger proportion of the flora before growth ceased for some reason. Even when the inoculum was only *ca.* 10–20 per piece the salmonellas increased more than 100-

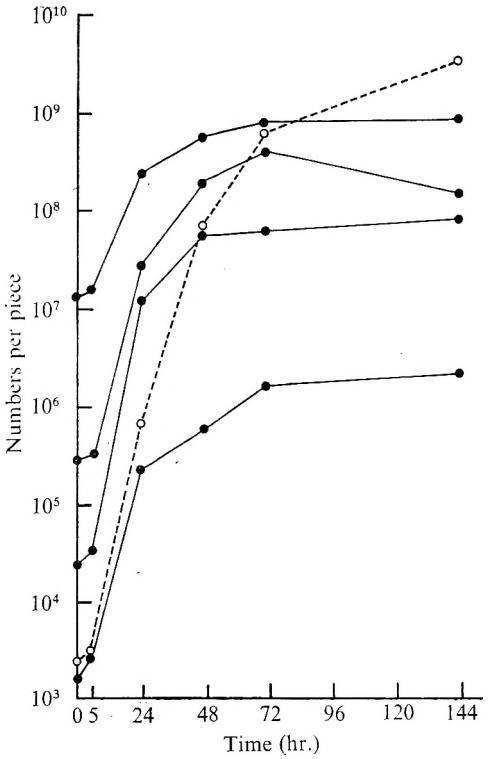

Fig. 3

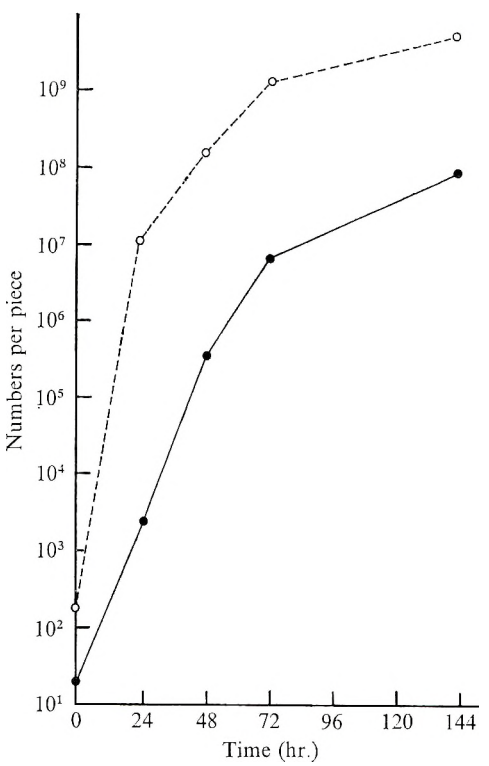

Fig. 4

Fig. 3. Growth of str^r mutants of *Salmonella enteritidis* NCTC5765 from various sizes of inoculum on 'low salt ham' at 22° C. The colony count on GYLA of total flora on samples carrying the smallest inoculum of salmonellas is shown, but all samples taken after the same incubation period gave comparable counts on GYLA. ●, *S. enteritidis* grown from various inoculum sizes; ○, Colony count on GYLA.

Fig. 4. Growth of a str^r mutant of *Salmonella enteritidis* NCTC5765 from an inoculum of *ca.* 20 organisms on 'low salt ham' at 22° C. ●, *S. enteritidis*; ○, Colony count on GYLA.

fold in 24 hr. at 22° C. and at 48 hr. the count was 10^4 times more than the inoculum (Fig. 4). Incubation of meat portions inoculated with *S. enteritidis* NCTC5765 at lower temperatures indicated that growth occurred at 17° C., although not until after 24 hr., but not at 5 or 10° C., where the number of salmonellas remained constant for at least 3 days (Fig. 5). When salmonellas were inoculated on to separate pieces of 'high salt ham' and incubated at 22° C. they grew slightly or not at all although the total flora increased considerably (Fig. 6).

Escherichia coli and *Proteus vulgaris*, two species that under natural conditions might be expected to be inoculated on to meat with salmonellas, were examined for their ability to affect growth of *S. enteritidis* NCTC5765. Pieces of 'low salt ham' inoculated with 3×10^6 *E. coli* 16A supported rapid growth of *S. enteritidis* (Fig. 7). Similar results were obtained with *E. coli* NCTC8984, which was chosen after studies in nutrient broth had shown that in the presence of *S. enteritidis* it could grow faster than the other four *E. coli* strains from NCTC. *P. vulgaris* (1×10^7) inoculated together with *S. enteritidis* NCTC8984 (5×10^3) into nutrient broth

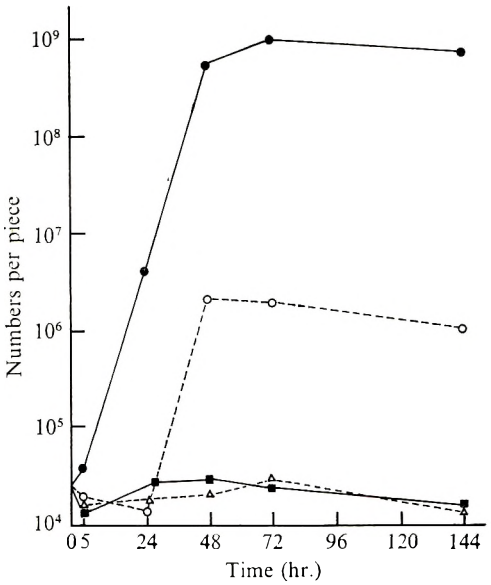

Fig. 5

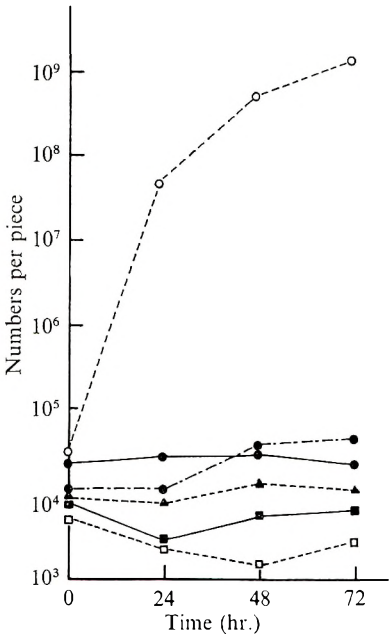

Fig. 6

Fig. 5. The effect of incubation temperature on growth of a str^r mutant of *Salmonella enteritidis* NCTC5765 on 'low salt ham'. ●, 22° C.; ○, 17° C.; △, 10° C.; ■, 5° C.

Fig. 6. Growth of str^r mutants of various salmonellas on 'high salt ham' at 22° C. Colony counts on GYLA of meat samples inoculated with *S. enteritidis* NCTC5765 are included. Meat samples inoculated with other salmonellas gave comparable results after the same incubation periods for colony counts on GYLA. ●——●, *S. enteritidis* NCTC5765; ●—·—·●, *S. typhimurium* 4718/70; ▲---▲, *S. newport* 3314/70; ■——■, *S. choleraesuis* NCTC5736; □---□, *S. pullorum* NCTC5776; ○---○, Colony counts on GYLA.

incubated at 22° C. also failed to restrict the growth of salmonellas, the counts being, after 24 hr.: *Salmonella*, 9×10^6; *Proteus*, $1{\cdot}5 \times 10^9$. In contrast *Staphylococcus aureus* is much inhibited by *E. coli* or *P. vulgaris* in broth, even when it outnumbers them by 100 to 1 in the inoculum (DiGiacinto & Frazier, 1966).

Effect of NaCl on growth of salmonellas

Static incubation at 22° C. of nutrient broth containing various concentrations of NaCl and inoculated with *ca.* 200/ml. *S. enteriditis* NCTC8984 showed that 6% NaCl, the concentration of NaCl in the 'high salt ham', inhibited the growth of the salmonellas (Fig. 8). Experiments with all 6 strains of *Salmonella* showed by turbidimetry that good growth occurred in nutrient broth containing 0·5% or 3% NaCl and that virtually no growth occurred in nutrient broth containing 5·5% NaCl within 24 hr.

Effect of $NaNO_2$ on growth of salmonellas

Turbidimetric assessment of growth of *S. enteritidis* NCTC8984 and *S. enteritidis* 4753/70 in nutrient broth incubated at 22° C. showed that nitrite at 100 p.p.m. added as $NaNO_2$ reduced by *ca.* 30% the growth of each strain at pH 5·0 in the

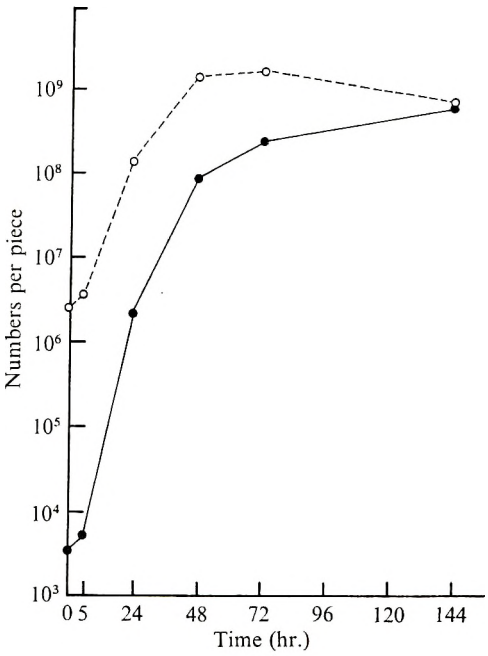

Fig. 7

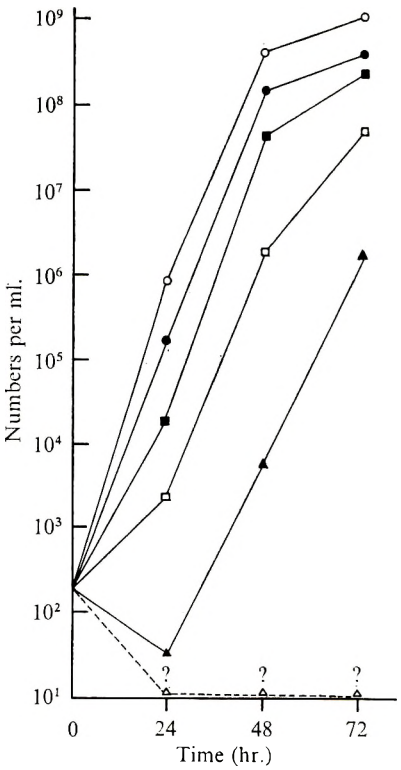

Fig. 8

Fig. 7. Growth at 22° C. of a str^r mutant of *Salmonella enteritidis* NCTC5765 and *Escherichia coli* 16A on 'low salt ham' when the inoculum contained 1000 times more *E. coli* than *S. enteritidis*. ●, *S. enteritidis*; ○, *E. coli*.

Fig. 8. Growth at 22° C. of *Salmonella enteritidis* NCTC5765 in nutrient broths containing various concentrations of NaCl. ○, 2% NaCl; ●, 3% NaCl; ■, 4% NaCl; □, 4·5% NaCl; ▲, 5% NaCl; △, 6% and 7% NaCl.

presence of 5% NaCl. At pH 5·0 in the presence of 3% NaCl the effect was slight and, as was expected, at pH 7·0 in the presence of either 3% or 5% NaCl the nitrite had no detectable inhibitory effect.

DISCUSSION

The use of streptomycin-resistant mutants to determine the conditions under which salmonellas could grow on cured pork was convenient and avoided confusion between inoculated salmonellas and any organisms initially present. The method would seem to have general applicability and has been used by Greenberg (1969) for study of population changes in the gut flora of flies. Drug resistance transfer between organisms from the inoculum and contaminants already present on the meat was not expected, because usually only resistance to concentrations up to 25 μg. streptomycin/ml. is conferred by an R factor (Weisblum & Davies, 1968), and did not appear to occur; all colonies on the isolation medium containing streptomycin resembled the colonies of the original str^r mutants and those tested by slide agglutination reacted like the original strains.

Choice of the method for inoculation of the meat samples was difficult because we wanted to deposit an inoculum of a known number of organisms without altering growth conditions from those normal on the product. We used 1 drop (0·02 ml.) prepared by diluting an overnight culture with 0·5 % (w/v) NaCl. This method seemed justifiable because under normal conditions contamination might occur via a drop of liquid (e.g. water dripping from thawing frozen meat; or blood dripping from meat) and because calculation indicated that the effect of the inoculum on the concentration of NaCl in the sample would be negligible. Thus, each sample of 'low salt ham' had a wet weight of 0·5 g., and contained *ca.* 0·35 g. H_2O. Addition of an inoculum in 0·02 ml. of 0·5 % (w/v) NaCl would reduce the content of NaCl by *ca.* 5 % e.g. from 2·87 g./100 g. H_2O to 2·73 g./100 g. H_2O.

Our results showed that 5 of the 6 salmonella strains examined grew rapidly at 22° C. on sliced cooked shoulder of pork with a NaCl content of *ca.* 2·8 g./100 g. H_2O, even when only a few organisms were inoculated and when other types of bacteria outnumbered the salmonellas, but that growth of salmonellas did not occur on a similar product with a NaCl content of *ca.* 6 g./100 g. H_2O. Experiments in nutrient broth with various concentrations of NaCl showed that little or no growth of salmonellas occurred in 3 days in the presence of more than 5 % NaCl. The results were comparable with those obtained by Vargues (1962; quoted by Jones, 1964) using *E. coli* and by Matches & Liston (1969) using *S. heidelberg*, *S. typhimurium* and *S. derby*, although these latter workers found greater tolerance to NaCl in nutrient broth at 37 than at 22° C. Using nutrient broth containing 1 % glucose and ham jelly containing 1 % glucose Koelensmid & van Rhee (1964) obtained good growth of salmonellas in 10 days at 20° C. but not at 5° C. with 6 % salt; with 8 % salt they obtained no growth at either temperature. Alford & Palumbo (1969) using nutrient broth apparently incubated for up to several weeks found that none of 23 salmonellas grew at 10° C. and pH 5·8 with 5 % NaCl but that at 20° C. all strains grew under these conditions and 6 strains grew with 8 % NaCl. At 30° C. 15 strains grew in 8 % NaCl. These workers also found that in ground pork at 10° C. and pH 5·75 no growth of salmonellas occurred with 5 % NaCl but some growth occurred with 3·5 % NaCl. They did not study growth on this material at higher temperatures. These various studies indicate that salmonellas are sensitive to the NaCl concentration present in the 'high salt ham' but that the sensitivity depends on temperature. Nitrite which is present in various amounts in cured meats is known to be bactericidal under certain conditions (Eddy & Ingram, 1956) but we did not investigate this in detail. Tests indicated that in nutrient broth nitrite was not very inhibitory at 100 p.p.m., while Koelensmid & van Rhee (1964) using ham jelly containing 500 p.p.m. $NaNO_2$ found growth of 3 of 4 salmonellas at 20° C., though this growth occurred only after several days during which time the nitrite concentration might have been reduced through reaction with amino groups.

The sizes of the populations of salmonellas reached on the 'low salt ham' after 24–48 hr., except in the case of *S. pullorum* which showed little growth, were comparable with $1{\cdot}5 \times 10^5$ organisms given by Wilson & Miles (1964) for minimum infective *S. newport* dose for adults. Nevertheless it must be appreciated that the

size of dose required to cause food poisoning may be much larger than this, depending on a variety of factors such as age of host and on characters of the strain of salmonella. We have made no attempt experimentally to determine infective doses of our strains or to assess either qualitatively or quantitatively the risk of contamination at the point of sale or in the home. However, salmonellas are common on raw meats (Hobbs, 1961; Prost & Riemann, 1967; Weissman & Carpenter, 1969) and salmonella food poisoning due to meat products constitutes a significant percentage of reported cases of food poisoning. We consider our examination indicates that cooked cured pork with a low NaCl content, once contaminated, is more likely to be a source of salmonella food poisoning than is a comparable product containing more NaCl.

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Determination of the colonization resistance of the digestive tract of individual mice

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SUMMARY

It has been shown that it is possible to investigate the colonization resistance in individual mice by determining the concentration of a certain contaminant (S.R.-*E. coli*) in the faeces during the first 4 days after contamination. Experimental contamination is contra-indicated in many cases such as in individuals with decreased resistance to infection. Particularly in this group, the value of the colonization resistance should be determined. It appeared to be possible to determine the colonization resistance in such individuals by quantitative biotyping of the Enterobacteriaceae species in the faeces on several consecutive days.

INTRODUCTION

The determination of colonization resistance (CR) of the digestive tract to pathogenic and potentially pathogenic (p.p.) bacteria in groups of animals is now a well-established technique that has contributed to the rational design of isolation methods in animal research.

We showed previously that the CR in mice is controlled by anaerobic bacteria which perform their activity directly by 'ecological interaction' and indirectly via the host (van der Waaij, Berghuis-de Vries & Lekkerkerk-van der Wees, 1971). An important aspect of the indirect activity is the stimulation of intestinal peristaltic activity by the microflora (Abrams & Bishop, 1966).

So far, colonization resistance has been measured by experimental contamination of mice and has been defined as the log of the oral dose of a specific bacterium that results in colonization of the digestive tract for 2 weeks or longer in 50% of a group of 20 animals (van der Waaij *et al.* 1971).

The measurement of CR in a group of mice is helpful in determining the extent to which isolation precautions must be provided in order to maintain the group free of potentially pathogenic bacteria. This technique does not provide specific information about the CR of individuals within a group. Such information is essential if the individual has a decreased resistance to infection and must be isolated in order to prevent development of infections from exogenous micro-organisms.

A possible method for determining individual CR was suggested by the finding that, after oral administration to mice of a single dose of an Enterobacteriaceae

species, the population density of these bacteria in the intestinal tract varied inversely with the CR. This study was undertaken to determine the nature of this inverse relationship. In addition, the concentration of other Enterobacteriaceae species in the faeces and the composition of the gram negative microflora was determined. This was done in order to investigate the possibility of the use of endogenous bacteria for the determination of CR. Total body irradiation, and antibiotic decontamination of the digestive tract were used alone or in combination in order to decrease the CR, while various contaminating doses of *Escherichia coli* were investigated.

MATERIALS AND METHODS

Mice

Conventional ND_2 female mice 8–12 weeks of age, weighing 28–35 g., were used.

Housing

The mice were housed one per cage in autoclaved macralon cages. The cages with antibiotic-treated animals were maintained in a laminar crossflow bench to prevent airborne contamination (van der Waaij & Andreas, 1971). Sterilized food and drinking water were supplied *ad libitum* to all animals.

Antibiotics

The mice that were decontaminated were treated with antibiotics. These were supplied *ad libitum* in drinking water containing 5 g. of neomycin, 5 g. of streptomycin, 5 g. of bacitracin and 0·1 g. of pimaricin/l. After the first week of treatment when stool and oral cultures were sterile, the concentration of streptomycin and bacitracin was reduced by 50 %, and use of neomycin was discontinued. Treatment with this low-dose regimen was continued throughout the experiment.

Oral contamination

The contaminating dose of a streptomycin-resistant (S.R.) strain of *Escherichia coli* was prepared by diluting an overnight broth culture with fresh broth to the desired concentration.

Conventional animals

Four groups of 8 mice were contaminated orally with doses of 10^3, 10^5, 10^7, 10^9 or 10^{11} S.R.-*E. coli* suspended in 0·1 ml. of broth. This procedure was repeated four times; 32 animals were thus exposed to each oral dose.

Antibiotic-treated animals

A contamination schedule identical with that for conventional animals was used. Two additional groups of 8 mice that received 10^2 bacteria were added. The bacteria were administered 10 days after the start of antibiotic treatment. The reasons for waiting 10 days were to assure 'physiologic stability' and complete elimination of neomycin from the g.i. tract (van der Waaij, 1969).

Irradiated animals

A contamination schedule identical with that for conventional mice was used. However, instead of 4 groups of 8 mice, only 2 groups were used for each dose. Contamination was performed at day 4 after irradiation.

Sampling

Inasmuch as the spread in the log of the concentration of S.R.-*E. coli* in faecal samples initially collected 3 times daily was very small (in no case more than 1 log), only one daily collection was used. After oral contamination, fresh faeces were collected on days 1, 2, 3, and 4. In the experiments with the unirradiated conventional mice, collections were also made on days 7 and 14. A 50 mg. sample of faeces was suspended in 0·5 ml. brain heart infusion broth (DIFCO). Subsequently, serial dilution was performed in trays in steps of 1 : 11. The trays contained 0·5 ml. of plain brain heart broth per cup or 0·5 ml. of broth to which streptomycin (100 μg./ml.) had been added. The streptomycin broth was employed when it was desired to isolate only streptomycin-resistant *E. coli*. Dilution was accomplished by the use of diluting loops (Flow labs) of 0·05 ml. capacity. Eight dilution steps routinely were carried out for each sample. When higher concentrations were expected, serial dilution was performed in 12 dilution steps. After overnight incubation at 37° C., the cultures were subcultured on Endo agar (DIFCO) for isolation, pure culturing, and biotyping.

Biotyping

With the help of 19 different fermentation reactions, the Enterobacteriaceae species isolated from the faeces of 4 conventional irradiated and 4 conventional unirradiated mice were typed. Details of the typing technique were described in an earlier publication (van der Waaij, Speltie & Vossen, 1972). Biotyping of the aerobic gram negative faecal bacteria was performed twice weekly for 4 subsequent weeks in the unirradiated mice and until death in the irradiated animals. Inventory of the faeces by biotyping the Enterobacteriaceae species isolated was started at the day on which the animals were experimentally contaminated. The mice belonged to the groups which received an oral dose of 10^5 S.R.-*E. coli* cells.

Biotypes which were isolated only once are designated as 'contaminations'; those which were isolated from two or more subsequent samples are indicated as 'colonizations'.

Irradiation

The animals received total body irradiation in perspex cages under conditions of maximal back scatter. Irradiation was performed with a Philips Muller X-ray machine at 300 kV, 10 mA with an HVL of 3 mm. Cu. The applied irradiation dose was 700 rad. For the conventional animals used in this study, this dose represents an LD 100. It results in severe bone marrow damage and in the first day(s) after irradiation some mucosal damage to the intestines (Gordon, Ruml, Hahne & Miller, 1955). Groups of 16 mice were contaminated with the same doses of S.R.-*E. coli* as were given to the unirradiated conventional animals. Contamination was

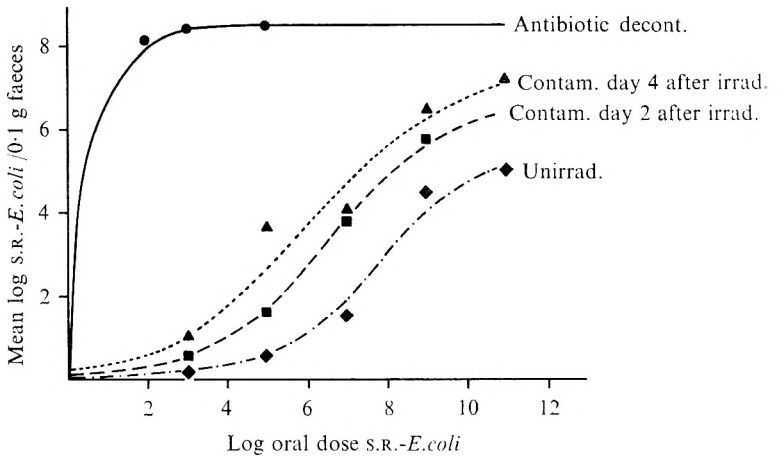

Fig. 1. Relation between the oral dose of S.R.-*E. coli* and its concentration in the faeces after contamination of antibiotically decontaminated, irradiated, and unirradiated conventional mice.

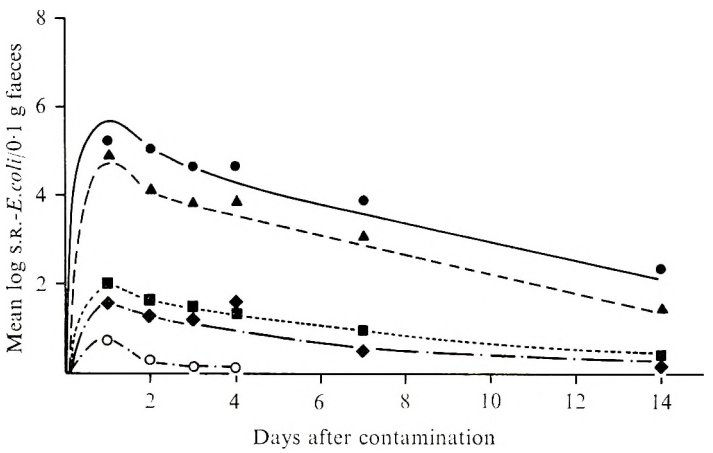

Fig. 2. Mean* faecal concentration of S.R.-*E. coli* at various intervals after contamination in conventional unirradiated mice. Oral dose of S.R.-*E. coli*: ●, 10^{11}; ▲, 10^{9}; ■, 10^{7}; ◆, 10^{5}; ○, 10^{3}. * 32 mice/dose.

performed on the second day after irradiation. In similar experiments, contamination was performed on day 4 after irradiation. The antibiotic-treated animals were irradiated under isolated conditions.

RESULTS

The mean faecal concentration of S.R.-*E. coli* during the first 4 days after contamination in conventional irradiated and unirradiated mice varied directly with the dose (Fig. 1). In the unirradiated animals, the peak concentration occurred within 1 day and was followed by a gradual reduction (Fig. 2). In the irradiated mice, a peak concentration at day 1 was only seen after the lowest dose of 10^3 bacteria (Fig. 3). After the contaminations with higher doses, no peaks were seen,

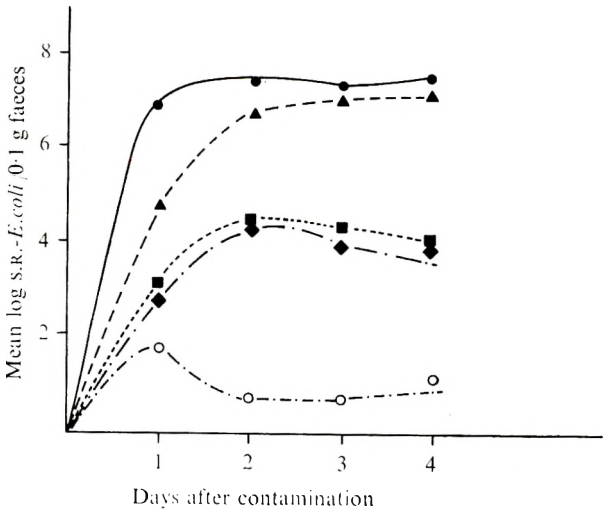

Fig. 3. Mean* faecal concentration of S.R.-*E. coli* at various intervals after contamination in conventional irradiated mice. Oral dose of S.R.-*E. coli*: ●, 10^{11}; ▲, 10^{9}; ■, 10^{7}; ◆, 10^{5}; ○, 10^{3}. * 16 mice/dose.

while considerably higher concentrations of bacteria /g. of faeces were seen than in the unirradiated mice.

The relation between the log of the oral dose of contaminating bacteria and the log of the mean concentration of S.R.-*E. coli* in the faeces is illustrated in Fig. 1. The mean concentrations are calculated from the data obtained in the first 4 days after contamination. This is the most likely period for colonization to occur even after low contaminating doses of bacteria (van der Waaij, de Vries & Lekkerkerk, 1972). Antibiotic-decontaminated mice were shown to have a concentration of approximately 10^{8} bacteria/0·1 g. of faeces following doses as low as 100 cells, whereas unirradiated conventional mice never exceeded a mean concentration of 10^{6} bacteria/0·1 g., even when 10^{11} bacteria were administered. Furthermore, at lower doses of 10^{3} S.R.-*E. coli* cells and, presumably even up to 10^{4} cells, no measurable concentrations were found in the faeces of most mice. The shape of the dose–faecal concentration curve for irradiated mice was similar to that for unirradiated mice, but the mean S.R.-*E. coli* concentrations in the faeces were higher. These results indicate that elimination of the microflora by antibiotic decontamination has a much more drastic effect on the CR than does lethal irradiation. For every interval after contamination (days 1, 2, 3, 4, 7 and 14), the percentage of positive samples and the concentrations of the contaminant in the samples were determined. These determinations were made without reference to the contaminating dose. For each of the 6 periods, a linear correlation was demonstrated between the mean log concentration of S.R.-*E. coli* in the faeces of individual animals within a group and the percentage of the group excreting S.R.-*E. coli* (Fig. 4). Inasmuch as the standard deviation of the mean log concentration of S.R.-*E. coli* in the faeces was shown to be very small (S.D. varied between 0·20 and 0·32) (Fig. 4), it is possible to relate individual concentrations to the percentage of animals excreting the contaminant. Fig. 4 also indicates that the level of CR, expressed as the percentage

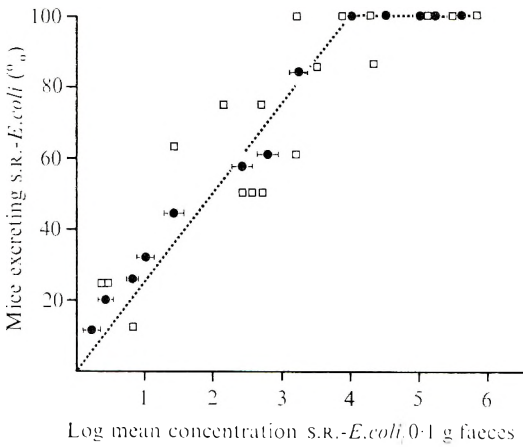

Fig. 4. Relation between the mean* concentration of S.R.-*E. coli* in the faeces 1–14 days after oral contamination and the percentage of mice that excreted the contaminant. * Unirradiated 32 mice/dose; irradiated 16 mice/dose. □, Mean irradiated mice (8 animals/group); ⊢●⊣, mean and S.D. unirradiated mice (80 animals/group).

Table 1. *Quantitative and qualitative results of biotyping of Enterobacteriaceae species following contamination with* S.R.-E. coli *in conventional irradiated and unirradiated mice**

Condition mice	Log oral dose S.R.-*E. coli*	Mean log conc./0·1 g. faeces in first 4 days: Contaminant (S.R.-*E. coli*)	Mean log conc./0·1 g. faeces in first 4 days: Other gram negative bacteria	Biotyping results gram negative bacteria: Mean no. cont./sample	Mean no. colon./sample	Colon./contam.
Conventional unirradiated	5	4·2	4·1	0·57	0·20	0·35
		4·1	3·5	0·14	0	0
		4·7	4·6	0·29	0	0
		4·5	4·3	0·49	0·20	0·40
Conventional contaminated at day 2 after irradiation	5	4·2	6·5	1·14	0·80	0·70
		5·2	7·1	0·85	0·80	0·94
		5·0	6·2	1·43	1·00	0·70
		4·7	6·1	0·57	0·60	1·05

* 4 animals/group.

of mice that excrete the contaminant is independent of the dose in which the contaminant was administered and of the time interval after contamination.

The biotyping results (Table 1) show a relation between the concentration of the various biotypes in the faeces and the mean concentration of the contaminant (S.R.-*E. coli*) found in the first 4 days after contamination. In unirradiated conventional mice, the average concentration of the various biotypes was of the same order of magnitude as the concentration in which S.R.-*E. coli* was found. In the irradiated animals, the mean concentration of the endogenous gram negative bacteria was generally higher than that of S.R.-*E. coli*. Since a constant relation

between the mean faecal concentrations of S.R.-*E. coli* in the first 4 days and at day 14 after contamination was seen in individual mice, it seems justified to draw conclusions with regard to CR from data obtained during the first days after contamination. The increased faecal concentrations of S.R.-*E. coli* and other biotypes in the irradiated animals indicate that their CR was decreased in comparison with unirradiated controls. The decreased CR following irradiation is also reflected in the mean number of 'contaminations' and 'colonizations' found during the observation period following experimental contamination of these mice with S.R.-*E. coli*. Although both groups were maintained under identical conventional conditions, the number of 'contaminations' found in the irradiated animals was considerably higher in the irradiated animals than in the unirradiated control animals. The number of 'colonizations' was also much higher in the irradiated group.

DISCUSSION

The results of these experiments indicate that the measurement of CR in individual animals is both feasible and practicable. The CR of an individual mouse can be directly expressed as the log concentration of a specific p.p. bacterial species found in the faeces 2 weeks after contamination. Since a constant relation was found between the concentration of S.R.-*E. coli* at day 14 and the concentration during the first days after contamination, apparently the concentration during the first days can be used as well. The results summarized in Figs. 2 and 3 show that low-dose contaminations with 10^3 S.R.-*E. coli* cells generally do not result in colonization of unirradiated conventional mice. It is apparent that, since the concentration can be used as a measure, the antibiotic decontaminated mice (Fig. 1) exhibit an extremely low CR, while conventional unirradiated animals have a high CR for contaminating doses up to 10^4 S.R.-*E. coli*. For higher oral doses, the CR of conventional mice decreases. Irradiated animals fall between the extremes of the antibiotic decontaminated and conventional unirradiated mice.

In the biotyping experiments (Table 1), in which the Enterobacteriaceae species colonizing the digestive tract of conventionally maintained mice were typed, great stability in the composition of the intestinal flora of conventional mice was found. In addition, it appears that our results with *E. coli* may have wider application with respect to the colonization of other Enterobacteriaceae species in conventional mice. The mean concentrations in which the various biotypes were isolated from the daily sampled faeces of the conventional unirradiated mice were in the same range as the S.R.-*E. coli* isolated when the animals had been contaminated with 10^5 cells. This could indicate that contaminations with gram negative rods from the environment via food and other sources generally do not exceed a dose of 10^5 bacteria. The increased number of 'contaminations' in the irradiated mice could indicate furthermore that a great number of contaminants from environmental sources do not 'take' in unirradiated mice. According to the results of experimental contamination with S.R.-*E. coli*, this difference in the occurrence of 'contaminations' between irradiated and unirradiated mice could be due to low dose contaminations. In that case, the dose range of contaminations from environmental

sources that result in noticeable concentrations for less than a week is apparently quite small and varies between 10^4–10^5 bacteria for unirradiated mice. The contamination dose that results in a 'contamination' is then extended in irradiated animals into the lower dose range of less than 10^4 bacteria. Our results, depicted in Figs. 1 and 3, may confirm this assumption. They indicate that irradiated animals become colonized after relatively low (10^3) oral doses of contaminating S.R.-*E. coli*. The decreased CR in irradiated mice is furthermore reflected in the increased concentrations of biotypes other than S.R.-*E. coli* as well as in the mean number of 'contaminations' and 'colonizations' (Table 1). While in the unirradiated animals the mean concentration of S.R.-*E. coli* was of the same range as that of other Enterobacteriaceae biotypes in the faeces, the concentration of S.R.-*E. coli* in irradiated mice was much lower than that of several faecal Enterobacteriaceae biotypes (Table 1). This cannot be attributed to increased contaminations from environmental sources after irradiation, because the unirradiated control animals maintained under the same environmental conditions did not show it. This 'selective' change in CR after irradiation could possibly be due to a decrease in the production of corresponding IgA antibodies in the gut. Bazin, Maldague & Heremans (1970) and Bazin *et al.* (1971) have described a considerable decrease in the number of IgA-producing cells in the intestines following lethal irradiation. The lack of sufficient intestinal antibodies preventing adherence and colonization (Freter, 1972; Williams & Gibbons, 1972) could then be responsible for the effect. Both authors conclude from their experiments that the prevention of bacterial adherence to the mucosa reduces the chance of colonization. Because surface epithelial cells are continually shed, colonization requires continuous reattachment; otherwise, the bacteria that tend to colonize are removed by mechanical factors such as secretions and peristaltic movement. To understand why some biotypes grow out in higher concentrations after irradiation than others, the following points could be of importance: first, the dose of S.R.-*E. coli* given appears to influence the concentration in which this contaminant is isolated from the faeces (i.e. colonizes the intestines) and second, different doses of different gram negative species are required to obtain the same faecal concentration (van der Waaij *et al.* 1971). The various Enterobacteriaceae species therefore will colonize the intestines in different concentrations most of the time. This is confirmed by our present quantitative biotyping experiments. Those biotypes that colonize the intestines in higher concentrations during the first days after irradiation may use up the available IgA more rapidly than those which colonize the intestines in lower concentrations. The available concentration of anti-S.R.-*E. coli* IgA could have been decreased less than that of IgA to several of the endogenously colonizing biotypes.

These findings imply that irradiated animals have a decreased CR. Such animals therefore must be carefully isolated to prevent exposure to contaminations inherent in the conventional laboratory animal environment, because once p.p. bacteria colonize, they form a potential danger for infection. Protective isolation should be considered mandatory for animals that have been decontaminated and irradiated in which the CR has decreased to extremely low values. Consequently, colonization occurs in high concentrations which implies the occurrence of spread

into lymphatic organs such as mesenteric lymph nodes and spleen (van der Waaij, de Vries & Lekkerkerk, 1972).

To measure CR in individuals such as human patients or monkeys with decreased resistance to infection, one must take advantage of the contaminations to which such individuals have been exposed in the conventional environment. Experimental oral contamination with a known dose of a particular p.p. bacterial species as in the present study is generally not possible in such individuals, as it involves a risk of infection caused by the contaminant. However, if the mechanism responsible for CR is similar in primates and mice, the measurement of CR could be accomplished by quantitative biotyping of one or more Enterobacteriaceae species isolated from faeces collected at 3 or 4 daily intervals such as has been done by Dankert (1973). The mean concentration of the typed bacteria is determined from the time related concentration curve. Typing of coliforms, klebsiella, and proteus species, as well as the determination of the concentration in which they are present in the faeces, will then give information concerning the CR of the intestinal tract to these gram negative bacteria. Determination of the concentration of the various Enterobacteriaceae species is not sufficient in itself, since different biotypes of the same species may subsequently determine the faecal concentration. Theoretically, a high CR can be misinterpreted as a low CR if the conclusion is based on the peak concentration following high dose daily contaminations with different bio- or sero-types of the same bacterial species.

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Serotypes of trachoma agent isolated in The Gambia: with an observation on the relation between serotype and morphology

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SUMMARY

Of 60 TRIC agents isolated from Gambian children with trachoma, 25 were serotype 1 and the remainder type 2. There was a pronounced difference in the proportions of these types in the two villages studied. In the village with a predominance of type 2 strains, TRIC agents remained confined to 2 adjacent compounds over a 14 month observation period. All 19 type 1 strains examined were characterized by the appearance in yolk sac smears of compact aggregates of elementary bodies; such aggregates were seen in only 2 of 35 type 2 strains, and may reflect a chemical difference in the surface of the elementary bodies or in a substance elaborated during their replication.

INTRODUCTION

The *Chlamydia* causing trachoma and inclusion conjunctivitis (TRIC agents) were first grouped into serotypes by the mouse toxicity prevention test (Bell, Snyder & Murray, 1959; Bell & Theobald, 1962; Chang, Wang & Grayston, 1962). This method was later superseded by indirect immunofluorescence techniques that gave results corresponding closely with those of the mouse test, and by means of which the same serotypes can be elicited. In general, patients with ophthalmic TRIC infection where trachoma is endemic yield types 1, 1b and 2 (McComb & Bell, 1967) which correspond respectively to types A, C and B (Alexander, Wang & Grayston, 1967). By contrast, types D, E and F of Alexander and his colleagues are usually isolated from patients with TRIC agent syndromes other than typical ophthalmic trachoma, such as inclusion conjunctivitis or genital tract infections.

The distribution of serotypes isolated from trachoma varies from country to country. Wang & Grayston (1971) commented that type B (2) strains have been isolated in all endemic areas, whereas types A (1) and the related C (1b) strains are not found in the same area; the exception is Saudi Arabia, where the incidences of types 1, 1b and 2 in 338 strains were 37 %, 25 % and 38 % respectively (Nichols,

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von Fritzinger & McComb, 1971). It was of interest to determine what serotypes are prevalent in The Gambia, whether the distribution varies in different places within the country, and whether these findings would be of use in epidemiological studies. This study formed part of a larger longitudinal investigation on trachoma in Gambian children, other aspects of which have been previously reported (Sowa, Collier & Sowa, 1971; Collier, Sowa & Sowa, 1972).

MATERIALS AND METHODS

Provenance of strains

The TRIC agents were isolated from the eyes of trachoma patients, nearly all children, in two villages: Salekini, the large village on the north bank of the River Gambia, in which our other studies were undertaken; and Berending, a small isolated village south of the river and about 100 km. to the west of Salekini. They were collected during 6 surveys made in the period 1969–70.

Isolation of TRIC agents

TRIC agents were isolated from conjunctival scrapings by inoculating the yolk sacs of 6-day chick embryos (Sowa, Sowa, Collier & Blyth, 1965). The precautions taken to avoid cross-contamination included a lapse of at least 1 hr. between the harvest of yolk sacs from different batches, during which the working area was thoroughly swabbed with alcohol. Furthermore, almost none of the isolates were passaged more than twice before they were serotyped. From heavily infected yolk sacs 50 % crude suspensions in phosphate-buffered saline (Dulbecco & Vogt, 1954) were made and stored at $-60°$ C. until use, when they were thawed and diluted to a 5 % concentration for making slide antigens.

Reference antigens

Dr Roger Nichols (Harvard School of Public Health) kindly provided the following strains in the form of 50 % crude yolk sac suspensions:

TRIC/1/ET/HAR-13/OT; TRIC/1b/SAU/HAR-32/OT;
TRIC/2/SAU/HAR-36/OT; TRIC/D/WAG/MRC-1/OT;
TRIC/E/USA-Cal-1/OT; TRIC/F/USA-Cal/Cal-9/ON.

The second element in the designation denotes the serotype (Gear, Gordon, Jones & Bell, 1963).

These suspensions were used as absorbing antigens and, diluted to 5 %, as slide antigens. The strains were used as received without further passage for all immunofluorescence tests done with donkey sera. For making the antigens used to immunize mice they were passaged once more in the chick embryo with stringent precautions to avoid cross-contamination.

The nomenclature of these serotypes has not yet been finally agreed; the workers at the Harvard School of Public Health designate the first three by numbers, whereas at the University of Washington, Seattle, letters are used for all serotypes. In this paper we shall use the Harvard system to designate serotypes 1, 1b and 2

identified by McComb & Bell (1967), and referred to by the Seattle workers as A, C and B respectively. We shall, however, use the Seattle system to refer to types D, E and F that were first described by Alexander *et al.* (1967).

Typing sera prepared in donkeys

Antisera to the various serotypes were kindly provided by Dr Roger Nichols, and were cross-absorbed in our laboratory according to the methods of Nichols & McComb (1964) and Nichols *et al.* (1971). After absorption they reacted specifically with their homologous antigens at the following dilutions: type 1 serum at 1/8; 1b at 1/4; 2 at 1/16; D at 1/60; E at 1/56; F at 1/96. When tested against prototype strains and a number of freshly isolated strains these sera gave results identical with those with control sera absorbed in Dr Nichols's laboratory. The anti-donkey fluorescein isothiocyanate (FITC) conjugate was also supplied by Dr Nichols and was used at a dilution of 1/14 determined by chessboard titration.

Preparation of antisera in mice

Immunizing antigens. Stored 50 % yolk sac suspensions of the reference strains were thawed in a water bath at 37° C., diluted to a concentration of 5 % with saline buffered with 0·01 M phosphate (PBS) (Fothergill, 1964) and homogenized for 1 min. at full speed in an M.S.E. blender. They were centrifuged at 800 *g* for 10 min. at 10° C.; the middle layer was diluted fivefold and stored in 5 ml. amounts at −60° C.

Antisera. These were prepared by a method based on that of Wang (1971); groups of 6 Swiss albino mice were injected intravenously with 0·5 ml. of antigen on days 0 and 4; they were bled on day 11 and the pooled sera were absorbed with normal yolk sac. The degree of cross-reactivity with heterologous antigens was higher than reported by Wang, and the sera were therefore absorbed in the same way as the donkey sera. After absorption, the mouse sera did not react even at low dilution with heterologous antigens and were used at the following final dilutions: types 1, 2 and F at 1/16; 1b, D and E at 1/8. The anti-mouse FITC conjugate was obtained from Flow Laboratories and was diluted 1/10 for use.

Indirect micro-immunofluorescence tests

The method was similar to that of Wang (1971).

Slide antigens. With a dip pen 'dots' of slide antigen were deposited on a slide aligned over a template; the dots were grouped so that one drop of serum dilution would cover 4 antigen spots. Each slide accommodated 48 antigen spots, including positive controls of known serotype and negative controls made from normal yolk sac suspension. After drying in air the slides were fixed in acetone for 10 min. at room temperature and stored at −60° C.

Staining method. Slide antigens were thawed and dried in a stream of air. Drops of typing sera, appropriately diluted in PBS, were applied to the antigen spots and the slides were incubated in a humid atmosphere at 37° C. for 30 min. They were then rinsed in PBS, immersed in fresh PBS for 10 min. and dried. The appropriate

Table 1. *Serotypes of TRIC agents isolated in two Gambian villages*

Village	No. of strains	Serotype 1	Serotype 2
Salekini	13	11	2
Berending	47	14	33
Totals	60	25	35

Table 2. *Distribution of TRIC serotypes in Berending*

Serotypes isolated	No. of families	No. of children yielding isolates
Type 1 only	3*	5
Types 1 and 2	2	10
Type 2 only	10	21

* All in one household (compound).

conjugate was then applied and the incubation and washing procedures were repeated. After a final wash in 75 ml. PBS containing 0·3 ml. of 1 % Evans blue as counterstain (Nichols & McComb, 1964) the slides were dried and mounted in glycerol:PBS, 9:1.

Ultraviolet microscopy. This was done as previously described for yolk sac slide antigens (Sowa *et al.* 1971), except that serum titration endpoints were taken as the first doubling dilution giving a fluorescence intensity of 2 with the set of graded density filters (Collier, 1968).

RESULTS

Comparison of results with donkey and mouse typing sera

Strains isolated during the first 5 surveys were typed with donkey sera; these reagents were exhausted by the time of the final survey, and recourse was had to the mouse sera that had been prepared for this contingency. Repeat tests with mouse sera on six type 1 and seven type 2 strains previously identified with donkey sera gave identical results.

Serotypes isolated

Table 1 shows that only serotypes 1 and 2 were isolated in the villages under study, and that neither type 1b, nor types D, E and F were detected. The relative proportions of types 1 and 2 varied in the two villages; in Salekini, 85 % of 13 strains were type 1, but in Berending, 70 % of 47 strains were type 2. In Berending, where the family distribution was studied, all the 21 children in ten families yielded only type 2 TRIC agents; the three families yielding only type 1 all lived in the same compound which was directly adjacent to the compound housing the two families yielding both types 1 and 2. (Table 2).

Morphological peculiarities of type 1 strains

We have previously described two strains of TRIC agent (G16 and G17) that differ from others in producing in yolk sac smears compact aggregates of elementary bodies that cluster densely around one or more vacuoles containing a

Table 3. *Association of elementary body aggregates (EBA) with serotype*

Provenance of strains	EBA present (+) or absent (−)	No. of strains		
		Type 1	Type 2	Totals
Salekini	+	10	2	12
	−	0	0	0
Berending	+	9	0	9
	−	0	33	33

carbohydrate, probably glycogen (Collier, 1959; Sowa & Collier, 1960). It was later noticed that these elementary body aggregates (EBA) were formed by about 25 % of strains isolated in The Gambia. They continued to appear in all subsequent chick embryo passages of these strains, and tended to be more numerous in chick embryos dying late (say 9–10 days) after inoculation. Table 3 shows the distribution by serotype of EBA in strains in which they were specifically looked for. There was a close correlation between the presence of these bodies and serotype, since of 21 strains producing them, 19 were type 1; they were found in only 2 of 35 type 2 strains.

DISCUSSION

This is the first report of the serotyping of a significant number of TRIC agents isolated from ophthalmic trachoma in The Gambia. The apparent absence of serotypes other than 1 and 2 from the villages under study is interesting, particularly since the first trachoma agent isolated in The Gambia proved to be type D (Wang & Grayston, 1971). This was surprising, because the strain in question (MRC-1/OT, formerly G1: Collier & Sowa, 1958) came from the eye of an 8-year-old girl with typical ophthalmic trachoma and is thus unlikely to have originated from a genital tract infection. The findings reported here suggest that the G1 strain is not typical of the generality of Gambian trachoma agents. The only other serotype detected by us was type F, isolated in Bathurst, the capital, from the eye of a newborn baby and from its father's urethra (Collier, Sowa & Sowa, 1969).

The finding of only 2 serological varieties of trachoma agent in the areas under study obviously limits the use of serotyping for researches on epidemiology in general and transmission in particular. It is however noteworthy that in Berending, despite the frequent movements of people between households, type 1 strains remained restricted to two adjacent compounds over a period of at least 14 months, suggesting that ophthalmic trachoma agents are not readily disseminated without close and frequent personal contact. Nichols and co-workers (1971) reported pronounced differences in the prevalence of types 1 and 2 in two Saudi Arabian villages only a few hundred yards apart and with considerable traffic between them; they considered that transmission of trachoma takes place largely within the family, and as far as they go our observations support this inference.

The close correlation between serotype and the appearance in yolk sac smears of dense aggregates of elementary bodies is particularly interesting. It must be stressed that these EBA are not merely local concentrations of free elementary

bodies but discrete structures that maintain their identity in suspension and resist centrifugation, freezing and thawing. Strains that produce them seem to do so indefinitely; we have observed them after 50 yolk sac passages of strain MRC-17/OT (formerly G17), in which they were first noticed and which later proved to be type 1 (Alexander *et al.* 1967). They can be readily identified in sections of chick embryo yolk sac infected with this strain (Sowa & Collier, 1960) but one of us (L.H.C.) has also observed similar objects in sections of yolk sac infected with MRC-4/ON (formerly LB4) at its second passage in chick embryos after isolation. This strain came from the eye of an English baby with neonatal inclusion conjunctivitis and is type 2; by contrast with the type 1 strains, yolk sac smears – as opposed to sections – do not contain EBA, which therefore appear to disrupt much more easily than those of the type 1 strains; but our experience with sections is too limited to support generalizations in this direction. Nevertheless, our observations on these Gambian strains do suggest that type 1 TRIC agents differ from type 2 in terms of the chemistry of the elementary body surface or of some substance elaborated during replication. It remains to be determined whether the binding material or the contents of the central vacuole – which seems to contain much carbohydrate – are endowed with serological specificity; if so, the EBA might provide a good source of type 1 antigen.

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A study of foot-and-mouth disease virus strains by complement fixation

I. A model for the fixation of complement by antigen/antibody mixtures

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SUMMARY

An examination was made of the relations between antigen, antibody and fixation of complement with foot-and-mouth disease virus (FMDV). It was found that complement fixation in this system follows the same principles as models developed in other antigen/antibody systems. The assumption that there is a relation of direct proportionality between the amount of complement fixed and the amount of antiserum reacting with constant antigen was found to be incorrect. An alternative method was proposed for the quantitative differentiation of FMDV strains by comparing the titres of an antiserum when reacting with optimum amounts of homologous or heterologous antigens.

INTRODUCTION

The use of complement fixation for the quantitative study of reactions between antigen and antibody was pioneered by Wadsworth and his co-workers (Wadsworth Maltaner & Maltaner, 1938*a*, *b*, *c*) and further developed by Mayer and others (Mayer, Osler, Bier & Heidelberger, 1948; Osler, Mayer & Heidelberger, 1948; Osler & Heidelberger, 1948*a*, *b*).

Fulton & Dumbell (1949) developed a method to study serological relations between strains of influenza virus. From the early work of Traub & Möhlmann (1946) and Brooksby, Galloway & Henderson (1948), which showed that foot-and-mouth disease virus (FMDV) strains could be differentiated by complement fixation, a method was developed for the measurement of serological differences between strains (Brooksby, 1952). The method was based on the comparison of the amount of complement fixed by homologous and heterologous mixtures using fixed antigen doses and varying the amount of antiserum and of complement in the mixtures.

In this paper, data are presented from experiments carried out to investigate the relations between antigen, antibody and fixation of complement in an FMDV system and, from the results obtained, a different approach is suggested for the differentiation of FMDV strains by complement fixation.

MATERIALS AND METHODS

Reagents

Diluent. Veronal-buffered saline (VBS) was prepared using complement-fixation buffer tablets (Oxoid Ltd, London)* with the addition of gelatin to 0·1 % w/v and was used for the dilution of all reagents used in the test.

Antigens. The virus strain used was FMDV type O, strain O_6 (OVI) except where a heterologous reaction was involved, when the strain was O_1 Lombardy (O_1 Lom). Virus was grown in baby hamster kidney (BHK 21) cell monolayers and the whole virus particles (140S antigen) were purified from the harvests by the method described by Brown & Cartwright (1963). The antigen was diluted in VBS and stored at 4° C. for a maximum of 1 week before use.

Antiserum. Guinea-pig antiserum was prepared against the OVI virus by the method described by Brooksby (1952). Before use the serum was inactivated for 30 min at 56° C.

Complement. The source of complement was pooled guinea-pig serum, adsorbed with washed sheep erythrocytes as described by Kabat & Mayer (1961), and stored in small volumes at −70° C. A fixed dilution of complement was used in the test to produce 75 to 80 % haemolysis in control mixtures.

Haemolytic system. Sheep erythrocytes (Wellcome Research Laboratories, Beckenham, England), stored in Alsever's solution, were washed according to the method of Kabat & Mayer (1961) and diluted in VBS.

The concentration of the erythrocyte suspension was estimated by lysing 1·0 ml. of the suspension with 9·0 ml. of water and measuring the optical density (O.D.) of the lysate at 541 nm. in a Pye Unicam SP500 Series II Spectrophotometer. The suspension was then appropriately diluted to give a lysate with an O.D. of $0{\cdot}300 \pm 0{\cdot}005$.

Rabbit anti-sheep haemolytic serum (Wellcome) was diluted 1/10 and stored in small volumes at −20° C. Erythrocytes were sensitized by slowly adding an equal volume of a 1/800 dilution of haemolytic serum and incubating in a water bath for 30 min. at 37° C. with occasional mixing. The sensitized erythrocyte suspension was then stored at 4° C. before use.

Protocol of the test

The reaction mixtures were prepared in disposable spectrophotometer cuvettes (Walter Sarstedt (U.K.) Ltd, Leicester) of 1 cm. path length and 3·5 ml. capacity. Reagents were added, using fixed volume micro-pipettes (Eppendorf Gerätebau Netheler u. Hinz GmbH, Hamburg, Germany) in the following order and volume: (i) antigen, 500 μl.; (ii) complement, 500 μl.; (iii) antiserum, 500 μl.; (iv) haemolytic system, 1000 μl.

In control mixtures, reagents were replaced as appropriate by the addition of the same volume of VBS in the same order.

The reaction mixtures were prepared in a water-bath at 0° C. They were then

* The constituents of the diluent are as follows: barbitone, 0·575 g./l.; calcium chloride, 0·028 g./l.; sodium chloride, 8·5 g./l.; barbitone soluble, 0·185 g./l.; magnesium chloride, 0·168 g./l.; pH approximately, 7·2.

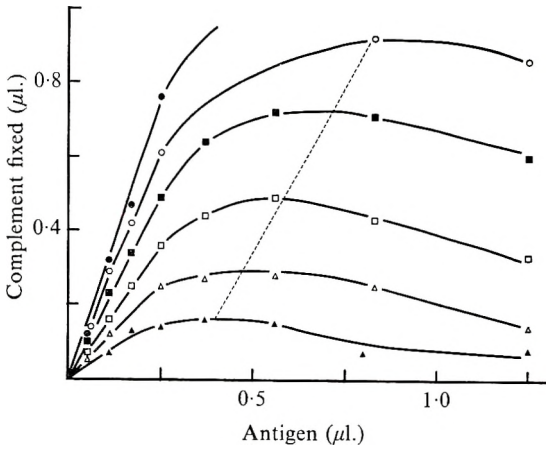

Fig. 1. The effect of varying the amount of antigen on the amount of complement fixed. Each curve represents a constant amount of antiserum. Serum (μl.): ●, 0·200; ○, 0·166; ■, 0·138; □, 0·115; △, 0·096; ▲, 0·080.

incubated in a water-bath for 30 min. at 37° C. before the addition of sensitized erythrocytes, which was followed by a further incubation for 45 min. at 37° C. The tubes were then chilled and centrifuged at 600 *g* for 10 min. to sediment unlysed cells. The O.D. of each mixture was then measured at 541 nm. and the proportion of lysis calculated relative to that in control tubes showing 100 % lysis (O.D. 0·600 ± 0·010).

The amount of complement fixed in each reaction mixture showing lysis between 10 % and 90 % was calculated, using the alternation formula of von Krogh (1916), viz.

$$\log x = \log k + (1/n) \log y/1 - y,$$

where x is the amount of complement remaining after primary fixation, k is the amount of complement required for 50 % lysis (1 C'H50), $1/n$ is a constant and y is the proportion of erythrocytes lysed.

The value of $1/n$ was established for the system as being 0·20 ± 0·02. The value of k was calculated using the same formula, from complement control mixtures where x was taken as the amount of complement added.

RESULTS

Variation of amount of antigen with constant antiserum

Fig. 1 shows the effects of varying the amount of antigen in a series of mixtures, each with the amount of antiserum constant. For any fixed amount of antiserum, the response to increasing antigen is at first a linear increase in the amount of complement fixed, followed by a region of maximum fixation where the proportion of antigen to antibody is optimal and then a range of relative antigen excess with a progressive decrease in fixation. Smaller amounts of antibody resulted in lower maximum fixation and a shallower slope in the zone of linear response.

The linear response is in the region of antiserum excess and under these circumstances the relation of amount of antigen to amount of complement fixed is directly

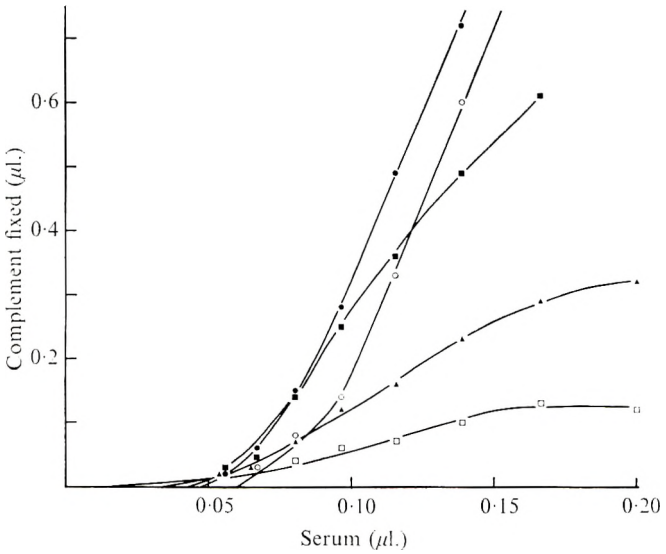

Fig. 2. The effect of varying the amount of antiserum on the amount of complement fixed. Each curve represents a constant amount of antigen. Antigen (μl.): ○—○, 1·25; ●, 0·56; ■, 0·25; ▲, 0·11; □, 0·05.

proportional. In the region of maximum fixation the slope is small, so that the optimum amount of antigen for a particular amount of serum is not well defined. However, the broken line in Fig. 1, which passes approximately through points of maximum fixation for each amount of serum, shows that the amount of antigen which is optimal increases with an increase in the amount of serum in the mixture.

Variation of amount of antiserum with constant antigen

Figs. 2 and 3 represent the effect of varying the amount of antiserum in a series of mixtures, each containing constant antigen. Fig. 2 is derived from the same experiment as Fig. 1. Comparison of these figures will reveal that the lines of constant antigen amounts of 0·25 μl., 0·11 μl. and 0·05 μl. in Fig. 2 represent antigen below the optimum over the range of amounts of serum used. While these curves are sigmoid, the lines representing 1·25 μl. and 0·56 μl. of antigen are linear over a large part of their range and are almost parallel.

Fig. 3, from a different experiment, shows lines of constant antigen amounts from 3·1 μl., which was close to optimal over the serum range used, to 15·6 μl., which represents a greater than fourfold antigen excess. The linear response to increasing serum with constant antigen is apparent, as is the parallelism of all but one of the lines.

It is evident from these figures that the relation of complement fixed to amount of serum with antigen constant, although linear, is not one of direct proportionality since the lines do not pass through the origin.

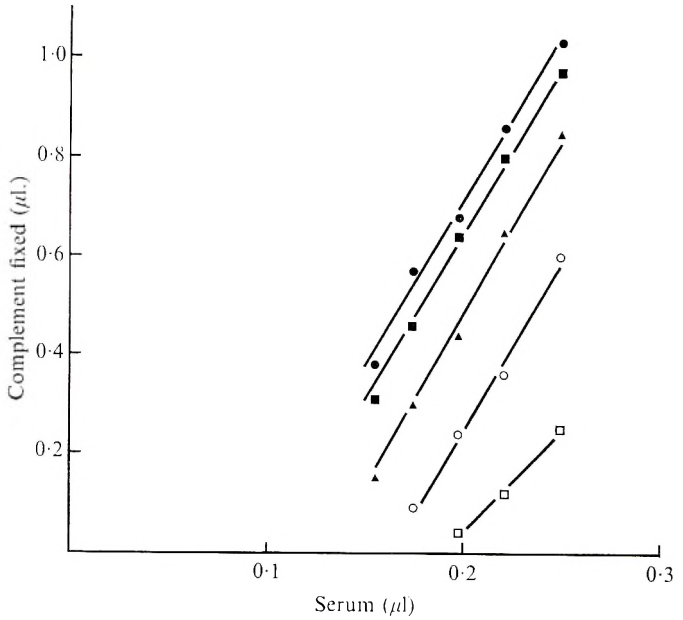

Fig. 3. The relation of the amount of antiserum to the amount of complement fixed. The lines represent constant antigen amounts over a range from close to optimal to a > fourfold antigen excess. Antigen (μl.): ●, 3·1; ■, 4·6; ▲, 6·9; ○, 10·4; □, 15·6.

Table 1. *Amounts of antigen and antiserum in optimum proportion mixtures, derived from Fig.* 1

Amount of antigen (μl.)	Amount of antiserum (μl.)	Ratio of antigen:antiserum
0·40	0·080	5·0
0·47	0·096	4·9
0·58	0·115	5·0
0·72	0·138	5·0
0·83	0·160	5·0

Variation of amounts of antigen and antiserum while maintaining them in optimal proportions

The broken line in Fig. 1, although inexact in position, is drawn to suggest the likelihood of a linear response between the amount of complement fixed and the amount of antigen which is optimum in each case. The ratio of antigen to antiserum in each optimum proportion mixture can be calculated from this (Table 1) and is apparently constant (the range of error in the ratios is approximately $\pm 0{\cdot}4$).

Fig. 4 is derived from Fig. 1 by plotting the points of maximum fixation of complement for each amount of antiserum. The graph shows a linear response between the amount of complement fixed and the amount of serum in each mixture. It demonstrates that, at least within this range of fixation, linear interpolation or extrapolation from two or more points could be used to ascertain the amount of serum required to fix a certain defined amount of complement. If this is taken as 0·5 C'H50 (0·69 μl. in Fig. 4), then 0·138 μl. of serum in a volume of 500 μl. is

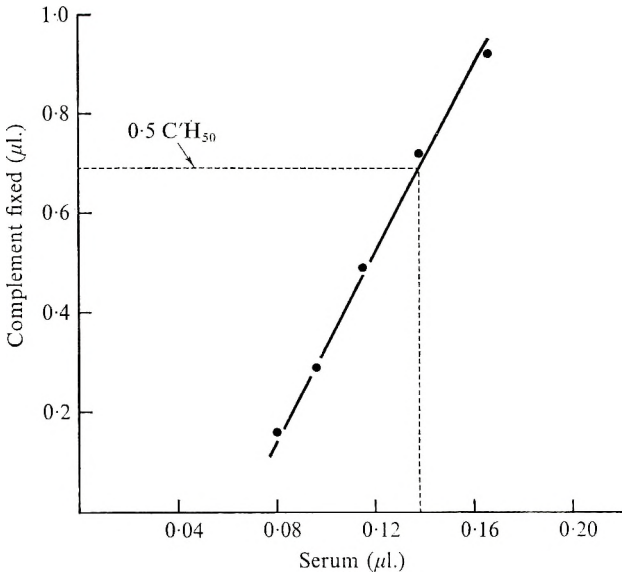

Fig. 4. The relation of the amount of antiserum to the amount of complement fixed in the presence of an optimal amount of antigen.

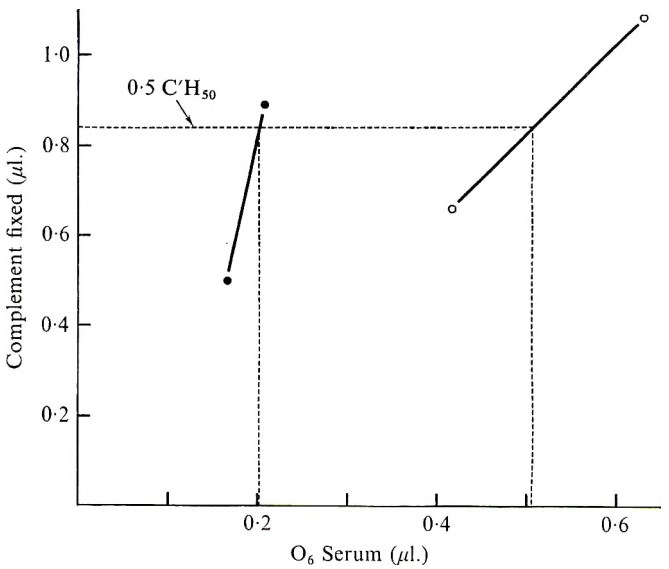

Fig. 5. The graphical estimation of titres of O_6 (OVI) antiserum with homologous and heterologous (O_1 Lom) antigens. Antigen: ●, O_6; ○, O_1.

required to fix this amount of complement with an optimal amount of antigen, i.e. the serum titre can be defined as 0·138/500 or 1/3623.

Reactions with homologous and heterologous antigens

Experiments carried out using a strain-heterologous antigen (O_1 Lom antigen with OVI antiserum) demonstrated that a similar pattern of fixation occurred but with a greater amount of antiserum required in the heterologous system, compared with the homologous system, for the same amount of complement

fixed. Homologous and heterologous antigens were tested over a suitable range of serial twofold dilutions against two levels of antiserum each, pretitrated to give maximum complement fixation in the desired range. The amount of complement fixed in each mixture was calculated and curves analogous to those of Fig. 1 were drawn for both antigens at each level of antiserum. From these curves the amounts of complement fixed with optimum antigen were determined and plotted (Fig. 5). The serum titres for the homologous and heterologous reactions were then found by linear interpolation as described above. Thus, the serum titre with homologous antigen (1/2475) was greater than with the heterologous antigen (1/990). It can also be seen from Fig. 5 that a greater proportional increase in the amount of antiserum is required in the heterologous reaction than in the homologous reaction for a similar increase in the amount of complement fixed.

DISCUSSION

The results presented in this paper suggest a model for complement fixation in this system which is consistent with those obtained by other workers, using more precise methods in more closely defined systems. Osler *et al.* (1948), using a single antigen/antibody system (pneumococcal capsular polysaccharide), presented data which bear a close resemblance to these results; and Shulman (1958) gave experimental and theoretical evidence for a similar model of complement fixation by complexes of antibody, quinidine as a haptene and platelets.

In each of these models there was evidence for lines of constant antigen concentration having regions of approximately parallel linear response, indicating that the relation between complement fixed and amount of antibody is not one of direct proportionality.

Bradish, Brooksby & Tsubahara (1960), studying the specificity of FMDV antigens, made the assumption that in constant antigen excess there was a direct relation between the amount of antibody and the amount of complement fixed. This was the basis for their calculation of complement-fixing activity and has been used by many workers since for subtype differentiation studies (Graves, 1960; Davie, 1964; Darbyshire, Hedger & Arrowsmith, 1972). However, the results from this paper suggest that the initial assumption was incorrect, so that this method of calculation of complement-fixing activity is invalid.

The estimation of a serum titre, as described above in relation to Fig. 4, is the basis of conventional chequerboard titrations. The linear response shown between the amount of complement fixed and the amount of immune complex is probably part of a sigmoid curve (Wallace, Osler & Mayer, 1950; de Almeida, Silverstein & Maltaner, 1952; Shulman, 1958) but the data in Fig. 4 suggest that in this system it is reasonable to assume this linearity over the range of fixation used in the test. The use of only two points on this line (as in Fig. 5) to determine a serum titre introduces the possibility of error in the estimate. However, if the error in position of the points is small and large extrapolation is avoided, the error in the estimate of the serum titre should in practice be acceptable. The reproducibility of results will be considered in a later paper (Forman, 1974).

As shown in Fig. 5, an antiserum will have a lower titre when reacting with a heterologous antigen than with its homologous antigen. It would appear that the expression of a difference in titres as a proportion (i.e. heterologous titre/homologous titre) is a valid method of measuring the relation between the two antigens. This expression was the basis of a technique for differentiation of FMDV strains by Roumiantzeff, Stellman & Dubouclard (1965) and Roumiantzeff, Dubouclard, Fontaine & Gilbert (1966) in a system using 5 C'H50 and a chequerboard titration of antigen and antiserum. The method described in this paper differs from that of Roumiantzeff *et al.* in the method of calculating serum titres and in using a lower dose of complement, enabling a more accurate measurement of residual complement in antigen/antibody mixtures compared with that in antigen, antiserum and complement control mixtures.

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A study of foot-and-mouth disease virus strains by complement fixation

II. A comparison of tube and microplate tests for the differentiation of strains

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(*Received* 28 *September* 1973)

SUMMARY

Several foot-and-mouth disease virus strains were examined by complement-fixation tests in microplates and in tubes. It was established that the two systems are comparable, although greater reproducibility is obtained with tube tests. While microplate tests are a satisfactory method for the differentiation of strains, tube tests provide a more precise method for the identification of small antigenic differences.

INTRODUCTION

In a previous paper (Forman, 1974) a model was presented for the fixation of complement by foot-and-mouth disease virus (FMDV) antigen/antibody mixtures. On the basis of results obtained from tests in tubes, it was suggested that virus strains could best be differentiated by the comparison of the titres of an antiserum when tested with homologous and heterologous antigens. The serum titre was expressed as the dilution of serum which would fix a defined amount of complement in the presence of an optimum amount of a particular antigen.

The principles demonstrated in that paper were applied to the differentiation of several FMDV strains by complement-fixation tests in both tubes and microplates.

MATERIALS AND METHODS

Reagents

The diluent (veronal-buffered saline), complement and haemolytic system were prepared in the manner previously described (Forman, 1974).

The antigens were the 140S particles of strains of FMDV grown as described in the previous paper (Forman, 1974) and purified by the method described by Brown & Cartwright (1963). The strains used were as follows: (i) two type O strains, O_6 (OVI) and O_1 Lombardy (O_1 Lom), which are classified by the World Reference Laboratory as distinct subtypes; (ii) two type A strains, A6003 and A6900, which had been compared by complement fixation previously by Guerche *et al.* (1972); (iii) three type Asia 1 strains, being a parent strain, Asia 1 C_2 and two derived strains, Asia 1 387 and Asia 1 415 which had been selected under mutagenic conditions for altered electrophoretic mobility. These strains had earlier undergone extensive comparative studies (Priston, 1972).

Antisera to the type O and type A strains were produced in guinea pigs in the manner described by Brooksby (1952), using live guinea-pig adapted virus. For

the Asia 1 strains, antisera were produced by inoculating guinea pigs once only with crude tissue culture harvests and bleeding them 28 days later.

Protocol of the tests

The tube tests were performed as described in the previous paper (Forman, 1974). To compare two strains, each antigen was used in a series of twofold dilutions over a predetermined range which would demonstrate an optimum dilution giving maximal fixation. Each serum was tested at two dilutions against each antigen and the amount of complement fixed with the optimum dilution of antigen at each dilution of serum was calculated. The titre of an antiserum with an antigen was then determined graphically, as previously described (Forman, 1974), as the dilution of serum which would fix 0·5 50 % haemolytic doses of complement (C'H50) with an optimum amount of antigen.

The microplate tests were performed in wells in plastic plates (Linbro–Biocult, Biocult Laboratories Ltd, Glasgow) in a final volume of 125 μl. Reagents were added in the following order and volume: (i) antigen, 25 μl.; (ii) complement, 25 μl.; (iii) antiserum, 25 μl.; (iv) haemolytic system, 50 μl.

The conditions used were designed as far as possible to parallel those used in tube tests. Thus, the reagents were added in the same order, all in 1/20 of the volume used in tubes. All dilutions were made in bottles and the reagents added to the plates with calibrated dropping pipettes. Mixtures were incubated in a hot-air incubator at 37° C. for 60 min. before the addition of sensitized erythrocytes, which was followed by a further incubation at 37° C. for 45 min. and then centrifugation at 600 *g* for 5 min. to sediment unlysed cells. It was established that primary fixation under the conditions used was similar to fixation in tubes after 30 min. in a water bath at 37° C. The tests were read by visual estimation of the sizes of deposited erythrocyte buttons.

The fixed dose of complement used in the microplate tests was 5 C'H50, established by pretitration in plates and equivalent in concentration to 5 C'H50 in the tube test. Control mixtures were included, with complement at 5, 2·5 and 1·25 C'H50 alone and with the antigens and antisera at all the dilutions used in the test.

To compare two virus strains in a microplate test, each antigen was used in a series of twofold dilutions, as in the tube tests, and tested against each antiserum in a series of 1·5-fold or twofold dilutions. The serum titre was determined as the dilution of serum which fixed four of the five C'H50 with an optimum amount of antigen, i.e. the highest dilution of antiserum in which 50 % of the sensitized erythrocytes remained unlysed. Where necessary, interpolation between two wells containing more than 50 % and less than 50 % unlysed cells was made by expressing the antiserum titre as the geometric mean of the two dilutions.

Calculation of strain differences

To obtain a quantitative estimate of the relation between two strains, the method used was that described by Ubertini *et al.* (1964) and popularly applied since then to FMDV strain differentiation.

Table 1. *Comparative complement-fixation tests in tubes and in microplates*

(*a*) Microplate tests – the numbers represent a visual score of the percentage of erythrocytes remaining unlysed: 4 = 100 %; 3 = 75 %; 2 = 50 %; 1 = 25 %.

			Antiserum (reciprocal of dilution)											
			A6003						A6900					
			16	32	64	128	256	512	16	32	64	128	256	512
Antigen (reciprocal of dilution)	A6003	32	4	4	4	4	—	—	4	4	4	—	—	—
		64	4	4	4	4	—	—	4	4	4	—	—	—
		128	4	4	4	4	2	—	4	4	4	2	—	—
		256	4	4	4	4	2	—	4	4	4	1	—	—
		512	2	2	2	2	—	—	—	—	—	—	—	—
	A6900	32	4	4	4	—	—	—	4	4	4	4	—	—
		64	4	4	4	—	—	—	4	4	4	4	1	—
		128	4	4	4	—	—	—	4	4	4	4	1	—
		256	3	1	1	—	—	—	2	3	3	2	—	—
		512	—	—	—	—	—	—	—	—	—	—	—	—

(*b*) Tube tests – the results are shown as the percentage of lysis in each mixture.

			Antiserum (reciprocal of dilution)							
			A6003				A6900			
			160	200	520	650	240	300	520	650
Antigen (reciprocal of dilution)	A6003	256			21·4	58·8	32·7	58·1		
		512			15·0	46·1	10·9	36·0		
		1024			27·3	54·7	17·7	35·7		
		2048			54·7	67·7	50·0	57·6		
		4096			70·4	76·4	71·0	73·4		
	A6900	128	23·7	50·8					42·4	68·7
		256	22·2	47·1					19·9	50·2
		512	32·5	53·7					15·7	40·7
		1024	55·2	66·2					39·1	49·8
		2048	71·0	75·4					64·5	69·2

For two antigens, A and B, and their respective antisera, a and b the following values are determined: r_1 (antiserum a) = Ba/Aa, where Ba represents the reciprocal of the titre of antiserum a with the heterologous antigen and Aa represents the reciprocal of the homologous titre of the same antiserum; similarly, r_2 (antiserum b) = Ab/Bb.

The antigenic relation between the strains (R) is then determined by the formula $R = 100\sqrt{r_1 . r_2}\,\%$.

RESULTS

Comparative fixation patterns in tube and microplate tests

In all the tests carried out, the pattern of fixation in microplates was comparable to that in tubes, as illustrated by Table 1. An optimum dilution of antigen was always demonstrable in tube tests. However, in microplate tests the antigen optimum was often represented by a range of dilutions, probably owing to the semi-quantitative nature of the end-point determinations in microplates.

Table 2. *Serum titres and values for* r *and* R *obtained in microplate tests*

(Parameters are shown as mean values with the range in brackets: (1), OVI *v.* O_1 Lom (three tests, two in duplicate); (2), A6003 *v.* A6900 (two tests, one in duplicate and one in triplicate).)

	Antigen	Antiserum	Serum titre	*r*	*R* (%)
(1)	OVI	OVI	1072 (994–1220)	0·54 (0·44–0·66)	40 (35–47)
	O_1 Lom	OVI	575 (441–661)		
	O_1 Lom	O_1 Lom	562 (397–661)	0·30 (0·30)	
	OVI	O_1 Lom	166 (118–196)		
(2)	A6003	A6003	272 (256–372)	0·36 (0·25–0·50)	42 (30–60)
	A6900	A6003	97 (91–102)		
	A6900	A6900	288 (256–324)	0·50 (0·35–0·71)	
	A6003	A6900	145 (128–162)		

Table 3. *Serum titres and values for* r *and* R *obtained in tube tests*

Parameters are shown as mean values with the range in brackets: (1), OVI *v.* O_1 Lom (4 tests); (2), A6003 *v.* A6900 (3 tests); (3), Asia 1 C_2 *v.* Asia 1 415 (3 tests); (4), Asia 1 C_2 *v.* Asia 1 387 (2 tests); (5), Asia 1 415 *v.* Asia 1 387 (2 tests).

	Antigen	Antiserum	Serum titre	*r*	*R* (%)
(1)	OVI	OVI	3548 (2564–3846)	0·37 (0·34–0·42)	30 (28–32)
	O_1 Lom	OVI	1318 (1097–1429)		
	O_1 Lom	O_1 Lom	1862 (1724–1887)	0·26 (0·23–0·26)	
	OVI	O_1 Lom	447 (427–485)		
(2)	A6003	A6003	589 (552–602)	0·28 (0·28–0·30)	37 (36–38)
	A6900	A6003	166 (153–178)		
	A6900	A6900	562 (538–658)	0·49 (0·47–0·52)	
	A6003	A6900	295 (281–311)		
(3)	Asia 1 C_2	Asia 1 C_2	195 (180–213)	1·00 (0·97–1·04)	83 (82–84)
	Asia 1 415	Asia 1 C_2	195 (174–221)		
	Asia 1 415	Asia 1 415	145 (127–152)	0·69 (0·67–0·71)	
	Asia 1 C_2	Asia 1 415	105 (94–122)		
(4)	Asia 1 C_2	Asia 1 C_2	195 (180–213)	0·95 (0·95)	101 (100–101)
	Asia 1 387	Asia 1 C_2	191 (177–202)		
	Asia 1 387	Asia 1 387	85 (79–100)	1·07 (1·06–1·08)	
	Asia 1 C_2	Asia 1 387	95 (86–108)		
(5)	Asia 1 415	Asia 1 415	145 (127–152)	0·64 (0·62–0·65)	80 (78–83)
	Asia 1 387	Asia 1 415	87 (81–94)		
	Asia 1 387	Asia 1 387	85 (79–100)	1·00 (0·99–1·00)	
	Asia 1 415	Asia 1 387	89 (78–99)		

Where inhibition of fixation in antigen excess was not observed in microplate tests, the demonstration of an optimum amount of antigen was accepted on the basis of at least two antigen dilutions indicating the same maximum antiserum end-point.

The results shown in Table 1 also indicate that in both systems the optimum dilution of an antigen was generally very similar for two different antisera, always being within 1 twofold interval for both. This was a consistent finding in all tube and microplate tests.

Reproducibility of results

Tables 2, 3 summarize the results of microplate and tube tests respectively. The ranges and mean values of the serum titres and of r and R were obtained from the values for individual tests and, where microplate tests were performed with replicates, the values for r and R were derived from all combinations of the appropriate replicate titres within each test.

The range of variation in the results was lower in tube tests than those in microplates. The maximum variation from a mean value for R, as a percentage of the mean, was 7 % in tube tests and 43 % in microplate tests. The values for R in comparative tests were slightly greater, but not significantly so, in microplate tests than in tube tests.

It is apparent that the range of variation in a serum titre in tube tests was greater than the variation in a value for R. Apart from the OVI antiserum of which a number of different pools were used, the greatest variation in serum titres in the tube tests was observed with the Asia 1 antisera. These exhibited large and variable pro-complementary effects and compensation for this by assuming that the activity was additive to that of complement was apparently inadequate. The values of r and R were affected much less by these errors, since they are proportional values.

In the microplate tests of OVI *v.* O_1 Lom, 1·5-fold serum dilutions were used. Since the range of variation of serum titres within tests was similar to those for tests of A6003 *v.* A6900, in which twofold serum dilutions were used, it would appear that there is no advantage in using the closer dilution interval.

Antigenic relations of the strains

From tests in tubes, the mean value for R between OVI and O_1 Lom was 30 % and between A6003 and A6900 was 37 %. In both these comparisons, antigenic differences were detectable with either serum but were quantitatively different, i.e. the values of r_1 and r_2 were unequal.

Two of the Asia 1 strains appeared to be identical (Asia 1 C_2 versus Asia 1 387, R = 101 %) and the third strain, Asia 1 415, was different but held a similar relation to the other two (Asia 1 C_2 *v.* Asia 1 415, R = 83 %, Asia 1 387 *v.* Asia 1 415, R = 80 %).

There was very little difference in the titres of either the Asia 1 C_2 or the Asia 1 387 antisera when tested against any of the three strains, so that antigenic differences between the strains was detectable only with the Asia 1 415 antiserum. The two values for r obtained with this antiserum were of the same order (Asia 1 415 *v.* Asia 1 C_2, r_1 = 0·69; Asia 1 415 *v.* Asia 1 387, r_1 = 0·64), which confirms the virtual identity of the Asia 1 C_2 and Asia 1 387 strains.

As the antisera to the Asia 1 strains were prepared using crude tissue culture antigens, it was possible that antibody to BHK cellular material could react with contaminating cellular debris. However, the method of antigen purification used should eliminate host material and there was no fixation of complement by any of the three antisera, tested at the lowest dilutions used, against a similarly purified heterologous (type O) antigen.

DISCUSSION

The comparative results of tube and microplate tests illustrate the essential difference between the two methods. Microplate tests, while simple and rapid to perform and to replicate, are considerably less reproducible than tube tests which, however, require considerable care in the preparation and pretitration of reagents. It would appear that the error in microplate tests would be quite acceptable for the routine differentiation of field strains of FMDV if the tests were sufficiently replicated. However, the tube test provides a sensitive and accurate method for the detection of small antigenic differences.

Guerche *et al.* (1972) found that the two type A strains, A6003 and A6900, could not be differentiated by complement fixation. Since they found that the two strains were immunologically distinct, they concluded that complement fixation was an unsatisfactory method for detecting immunological variants. These authors used a complement-fixation test in tubes in which the concentrations of antigen, antiserum and complement were all varied. Their results were not entirely consistent with the model of fixation described previously (Forman, 1974) but this could be explained by their use of crude tissue culture harvests as antigens, since these will contain at least three complement-fixing antigens, probably at different concentrations and with different strain specificities (unpublished data). The value for R determined in this study ($R = 37\,\%$) was of a similar order to that of the two type O strains which are accepted as different sub-types (OVI *v.* O_1 Lom, $R = 30\,\%$). It can only be concluded from this that the two strains, A6003 and A6900, are serologically distinct and this is consistent with the finding of Guerche *et al.* (1972) that the two strains are immunologically different.

The differences between the type Asia 1 strain 415 and the two other strains, C_2 and 387, were small but reproducible and in the light of the interrelations between the three strains would appear to be significant. It was demonstrated that the close similarities were not the result of fixation of complement by contaminating non-viral antigens. Nor is it likely that viral antigens other than 140S were reacting, since other unpublished work suggests that such antigens – in particular, 12S sub-units resulting from degradation of the virion – are present in such preparations in only very small amounts. These differences could, however, not be correlated with any of the other criteria by which the strains have been compared (R. Priston, 1972; work to be published).

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The effect of obstructions and thermals in laminar-flow systems

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SUMMARY

The influence of obstructions and thermals on the air flow in unidirectional or laminar flow systems was studied with special reference to operating rooms. It was shown that thermals induced in the operating rooms would have little influence in the normal laminar-flow system. The importance however of obstructions such as operating lamps and personnel was shown.

INTRODUCTION

At the present time, the air conditioning systems of the vast majority of operating rooms are being designed and built to conventional air-conditioning principles. These principles are embodied in the M.R.C. report on the air conditioning of operating theatres (Report 1962), and more recently in the Joint Working Party Report on the Ventilation in Operating Suites, (M.R.C. Report 1972).

Before the development of the laminar-flow principle of ventilation, work had been carried out in the conventionally ventilated operating-rooms to increase their efficiency in removing bacteria. Blowers & Crew (1960) studied, under experimental conditions, two different methods of distributing air to the operating room, namely uniform mixing with turbulence and downward displacement with a minimum of turbulence. They concluded on the evidence available at that time that the displacement system was best, and this they found to be best obtained with a finely perforated ceiling. This method was obviously a forerunner of the laminar-flow system but was shown to be ineffective owing to the use of low volumes of air 0·56 $m.^3/s.$ (1200 $ft.^3/min.$) and the turbulence produced by convection and staff movements (Stanley, Shorter & Cousins, 1964; Lidwell, Richards & Polakoff, 1967).

Although it is possible, with conventional ventilation systems, to make a considerable reduction in the number of bacteria in the air compared with those in a room with no ventilation at all, there is still a substantial number of bacteria present. In order to realize the hope that a reduction in the airborne bacteria in operating-rooms will be associated with a drop in wound sepsis, investigations have been made into ultra-clean environment for use in operating-rooms (Charnley, 1964; Whitcomb & Clapper, 1966).

A large number of systems of this type are now being installed throughout the world but it will be a long time before they are up to the standard of conventional

systems as many questions require to be answered. Two such questions are related to the effect of obstructions and of thermals in laminar flow. Although unidirectional flow has an advantage over turbulent flow in that bacteria produced should be swept away immediately they are generated, the effect of obstructions and thermals would tend to make the laminar flow into a turbulent one.

METHODS AND RESULTS

In carrying out this research we have used three techniques which may be of interest to those involved in the study of air flow. The flow of unidirectional air is often represented in diagrams by arrows or straight lines which frequently have little resemblance to the actual flow. This type of diagrammatic visualization arises often from the sheer lack of knowledge as to what is actually happening in a given situation. We describe therefore three techniques which may be useful to other investigators.

Schlieren photography

Hot air rising from an object hotter than the surrounding atmosphere is lower in density than the air around it. As the refractive index varies with density, light passing through the hot air will be refracted. By use of suitable optical techniques this effect can be used to show up the hot air round an object.

This technique to show up differences in density is not new and was used by the German glass industry to show up the 'Schlieren' or flaws in their glass. It has been scientifically developed by workers in the field of aeronautics and these methods are described in a comprehensive review by Holder & North (1963) but the use of this system to study heat flow round humans is described by Lewis *et al.* (1969). The method we have employed is similar to that used by Lewis and his colleagues.

The light source in our system was a high-intensity film projector bulb which was brought into focus by a condenser onto a pin hole which was at the focal distance from a high quality 12″ diameter mirror. The parallel light beam then passed through a 1 ft. diameter hole in the partition of the laminar flow system and out through a similar hole in the other wall. Any object to be studied was placed in the light beam in this area. The light beam was then focused by a second mirror on to a pinhole and photographs taken by a single-lens reflex camera without the lens. By use of this camera the shutter speed was easily obtained for a given type of film. Any hot air rising from an object in the parallel beam would cause refraction of the light rays which would then fail to pass through the pinhole and be shown as dark areas in the photograph.

In order to study the effect of heat rising from a hot body, we chose to examine one pod of the lamp (Drayton Castle 'Daystar') which we had used in our laminar flow operating room (Whyte, Shaw & Barnes, 1971). This was one pod of a four-pod lamp assembly. This lighting pod, because of the quartz-halogen system, used 250 watts as compared with about 350 watts for a complete conventional operating lamp and the 100 watts of sensible heat produced from a surgeon working fairly hard.

It may therefore be seen that the thermal effect of this lamp is likely to be the most intense of any heat source in an operating theatre and if the unidirectional flow is capable of overcoming these adverse effects it is likely to overcome all others. This is confirmed by measurements of velocity we took with a hot wire anemometer which showed that the velocity of the air rising from a pod of our operating lamp could be up to 0·6 m./s. (120 ft./min.) whereas it was only 0·2 m./s. (40 ft./min.) from a person.

These effects were investigated in the case of the lamp by Schlieren photography and in the case of a man by bubble generators.

Plate 1*a* shows the heat rising from the lamp in still air and Plate 1*b*–1*f* show the effect of a downflow of air of velocity, 0·1, 0·2, 0·3, 0·4 and 0·5 m./s. Shown in Plate 2*a*–2*f* is the effect of a crossflow of unidirectional air of velocity 0, 0·1, 0·2, 0·3, 0·4 and 0·5 m./s. There was a certain amount of variability in the effect of air at a given velocity and the photographs shown are typical of what happens but the same effect could occur on a small percentage of times at a velocity 0·1 m./s. greater or less than the given speed.

Bubble generator technique

It is normal practice to show the flow of air by use of smoke. This technique however has severe limitations, in that smoke quickly diffuses and also fails to give any indication as to the relative air speeds. It is also true that although smoke tests are of use to the person who is actually using them, they are of little use for permanent records of the observed air flow. In order to study and report the flow of air in different laminar-flow conditions we adopted a method recently developed by the National Institute of Agricultural Engineering in Britain, and now fully described by Carpenter & Moulsley (1972). This system uses an air supply mixed with helium and a simple bubble generator* to produce a flow of neutral-buoyancy detergent-bubbles of 4 mm. average diameter. We are also aware of a similar system produced in the U.S.A. by Sage Action, Inc., New York, and have been favourably impressed by the reliability of the two systems.

Carpenter and Moulsley advocated a relatively elaborate camera shutter-system which shows the direction of flow of the bubble as a series of dashes diminishing in size away from the direction of movement. This system is necessary where the direction of flow is arbitrary or unknown, but fortunately this is not so with unidirectional flow. It was only necessary in our case to photograph the bubble mass at a time exposure of 1 sec. to achieve photographs of the type shown in Plate 3*a*, *b*, and Plate 4*a*, *b*.

The original light source we used was a series of spot lights set up in a duct, the light being projected through a slit facing the flow of air to be studied. In the case of the obstruction being large and cutting off the light illuminating the area behind it, a further light source was added at the air supply and facing the direction of the air movement. We have now found a single fog lamp on each side of the object to be successful. They have a narrow light beam the intensity of which can be

* Further details about this bubble generator are available from the National Institute of Agricultural Engineering, Silsoe, Bedford, U.K.

controlled either by blanking off the light beam or by a variable transformer and they also have a small surface area and therefore do not disturb the air flow. The air movement bubbles were illuminated and the background kept dark with a backcloth. Two or three bubble generators were used in our studies and it may be seen that the bubble movement is shown up as a streak, its length being proportional to the velocity of the streamline. Knowing the actual length of the bubble streak by reference to a known object size in the photograph and the exposure time of the camera, the velocity of the air stream at a particular point may be ascertained.

Shown in Plate 3*a* is the airflow (0·4 m./s.) round a lamp 1 m. in diameter. This type of lamp is typical of those used in Europe and this shape of lamp is probably installed in over 99 % of the operating rooms in Britain. It may be seen from the direction of the bubbles and the lower velocity of those present at the lamp face that a large area of stagnancy exists behind the lamp. Plate 3*b* shows the way that the air flow is not disrupted when the lighting surface is parallel to the air stream.

Plate 4*a* is a bubble generator photograph of the lighting system we have adopted for our laminar-flow operating-room. This is a Drayton Castle 'Daystar' lamp which has two of the six pods of the lamp removed. The removal of two pods gives a lamp with satisfactory lighting for the surgeon and a better airflow pattern. It should however be realized that this lamp is asymmetrical and the bubbles are generated in one plane so that this photograph gives a better than average picture of the airflow pattern round the light as quite a number of the bubbles are passing, without resistance, through the centre of the lamp.

The photographs shown in Plates 3*a*, *b* and 4*a* are very similar to those obtained by Lidwell (1971) when using smoke trails and a model to investigate airflow in unidirectional flow systems.

It should also be noted that Plates 3*a*, *b* and 4*a* were of lamps in a horizontal air flow and in this flow the stream lines will converge again after passing round the light. However in the case of vertical flow the floor will tend to counteract this effect so that a larger stagnant area will result.

It may be seen in Plate 4*b* that the thermal effect from a person is overcome in a horizontal airflow of about 0·4 m./s. This is to be expected as the heat output of a human is not as great as one of the tested pods. It should be noted, however, that there is considerable turbulence behind a person and this appears to us to be a major factor in reducing the efficiency of laminar-flow systems.

Smoke challenge test

It should be realized that what is significant about the above bubble generator photographs is not the fact that the air downstream of an object is turbulent, or even stagnant, but that any contamination generated in this area, e.g. by a surgeon, would linger and make the area one of gross contamination. If there was no contaminating source, then the turbulence would be of little consequence. In order to illustrate this, observations were made of the route taken by smoke released in the highly turbulent zone after the lamp. A simple smoke source was

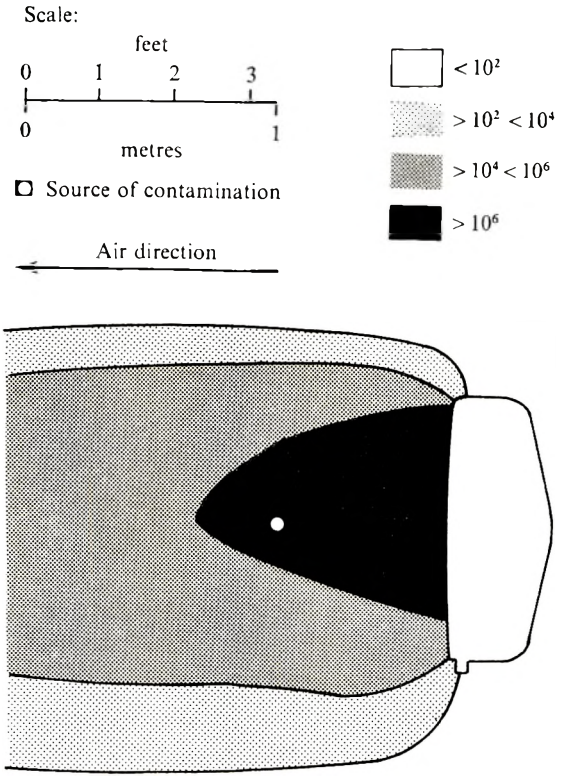

Fig. 1. Diagram showing the backflow of contamination from a source 0·8 m. from a large lamp.

used – a cigarette. This produced a surprisingly uniform source of smoke which, although it initially tended to rise, gave very reproducable results of the type illustrated in Fig. 1. Samples of air were analysed by a Royco Particle Sampler in a given plane at 20 cm. intervals and zones of equal contamination determined. These zones were expressed in concentrations per cubic foot of particles $\geqslant 0{\cdot}5\ \mu$m. The zones were $< 10^2$, between 10^2 and 10^4, between 10^4 and 10^6 and $> 10^6$ particles per cubic foot. These lines of equal contamination are known as isopleths.

Fig. 1 is an isopleth diagram demonstrating the effect of a 1 m. diameter operating lamp on a contaminating source placed 0·8 m. (30 in.) away from it. The contamination, instead of being carried forward, is actually drawn back into the stagnant area to give a zone of high contamination. If the contamination source is placed a further 0·6 m. (24 in.) away from the lamp, one would expect from the bubble generator photographs that the contamination would all now be blown forward. This in fact did not happen, as shown in Fig. 2. Although a reasonable percentage of it did move forward a surprising amount found its way back up to the lamp.

Fig. 3 shows the advantage of the smaller podded lamp. The contamination was 0·8 m. (30 in.) away and therefore similar to Fig. 1, but highlights the fact that even using this type of lamp, zones of high concentration can occur as seen by the flow back of contamination.

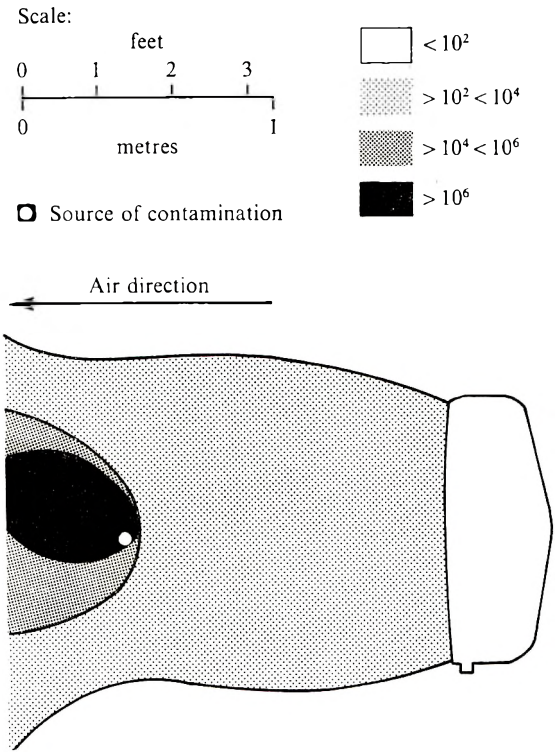

Fig. 2. Diagram showing the small amount of backflow and stagnancy from a source 1·4 m. from a large lamp.

DISCUSSION AND CONCLUSIONS

Unidirectional or laminar flow has an advantage over conventional turbulent ventilation systems in that the contamination generated is swept immediately away instead of being diluted. However several factors could tend to destroy the unidirectional nature of the airflow and negate its inherent advantages. One of these factors is the effect of thermals, the other that of obstructions.

Effect of thermals

Discussion of the conclusions reached in this paper with regard to thermal effects may be divided into two parts – one part concerned with the reaction of hot objects in an operating theatre to the down-flow of unidirectional air and the other with a cross-flow of air.

Hot air rising from the operating-lamp and people in an operating-room will tend to disrupt the downward flow of sterile air which would normally wash away the contamination produced in the area of the wound. We have studied the downward velocity that is required to overcome this effect from what must be the most intense source of heat likely to be found in an operating theatre, namely a pod of the Drayton Castle 'Daystar Lamp'. By use of Schlieren photography it has been demonstrated that the upward thermals are overcome at downwards air speeds of 0·3 to 0·4 m./s. (60–80 ft./min.). Smaller heat sources will of course

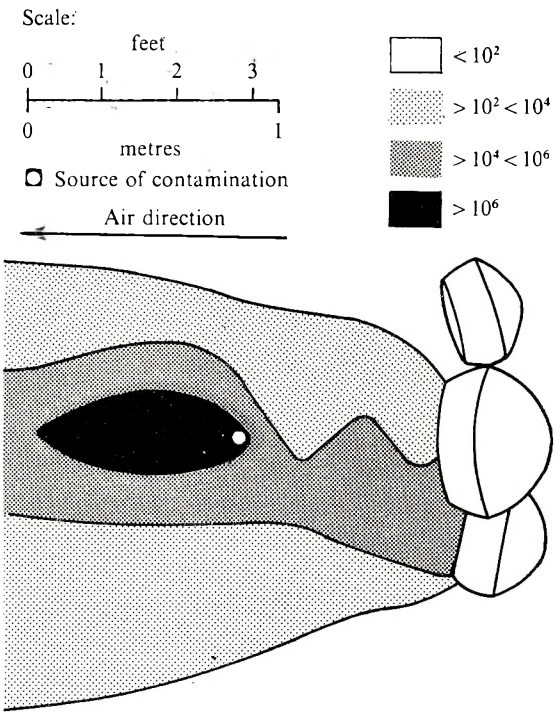

Fig. 3. Diagram showing much less backflow of contamination from a source 0·8 m. from a skeletal lamp.

require a lower velocity. This speed of between 0·3 and 0·4 m./s. coincides with the velocity we have advocated for laminar-flow operating rooms from studying the contamination concentrations at different velocities during operations in a down-flow or crossflow laminar-flow system. (Whyte, Shaw & Barnes, 1973).

The results obtained from the crossflow of air require fuller interpretation as there is no point at which the heat rising was completely overcome. Because the two forces (thermal and air velocity) were at right angles to each other the resultant thermal force of the heat rising from the lamp was up and in the direction of the air. As the air rose, however, it would be quickly overcome by further air velocity forces and turned in the direction of the horizontal air supply. Unfortunately owing to the small field of view of the Schlieren system it was not possible to see this effect but bubble generator studies which are not reproduced here demonstrated that at normal velocities, i.e. over 0·3–0·4 m./s., there was little discernable difference between the airflow round the lamp when it was on, and therefore hot, and when it was off. The main factor influencing the airflow was the obstruction by the lamp.

These above facts were confirmed by observation of airflow around a man where the thermal effects were easily overcome but the disruption of flow was caused by his obstruction and movement.

In conclusion it would appear that thermal effect may be discounted in laminar-air flow in an operating theatre.

Effect of obstructions

One of the most significant obstructions in an operating room is the operating theatre lamp, and we have studied this effect by two methods which may be of use to others. Using these two methods we hope we have shown that when a lamp with a large surface area is used there is a considerable stagnant area behind the lamp. The stagnant area would not be a problem if this air was not in contact with any source of bacterial dispersion, or if the area was not required to be sterile. This can be achieved in many types of surgery with a knowledge of what is happening to the air flow and how to overcome it by judicial siting of the lamp. Most people, however, in operating rooms have not the experience to do this, and it is therefore necessary to overcome this problem. An over emphasis, however, of this problem can lead to solutions such as the placing of lamps outside the air flow. This would normally remove the control of the lighting from the surgeon and use of the currently available equipment could result in a poor system which is more expensive. We therefore advocate at the present time an in-flow system with lamps of small enough diameter to produce turbulence which will not come into contact with the operating field. The benefits are shown in the use of our modified Drayton-Castle 'Daystar' lamp, but we are also aware of several other operating theatre light manufacturers who produce lighting systems which would also be suitable. The need for small operating lamps is far greater in down flow systems of ventilation, where the lamp may be in a position over the critical area of the wound and cause a stagnant area with a high concentration of bacteria.

In the case of cross flow, however, the need for modified lighting is much less, as the chance of the lamp being in such a position as to cause a stagnant area at the wound is more remote. However, use of lamps with a smaller surface area could do nothing but help the air flow pattern.

In a fast developing field such as that involved in the production of ultra-clean ventilation systems, many new ideas will be produced which will no doubt supersede ours, but it is hoped that the methods outlined above will help to evaluate these systems.

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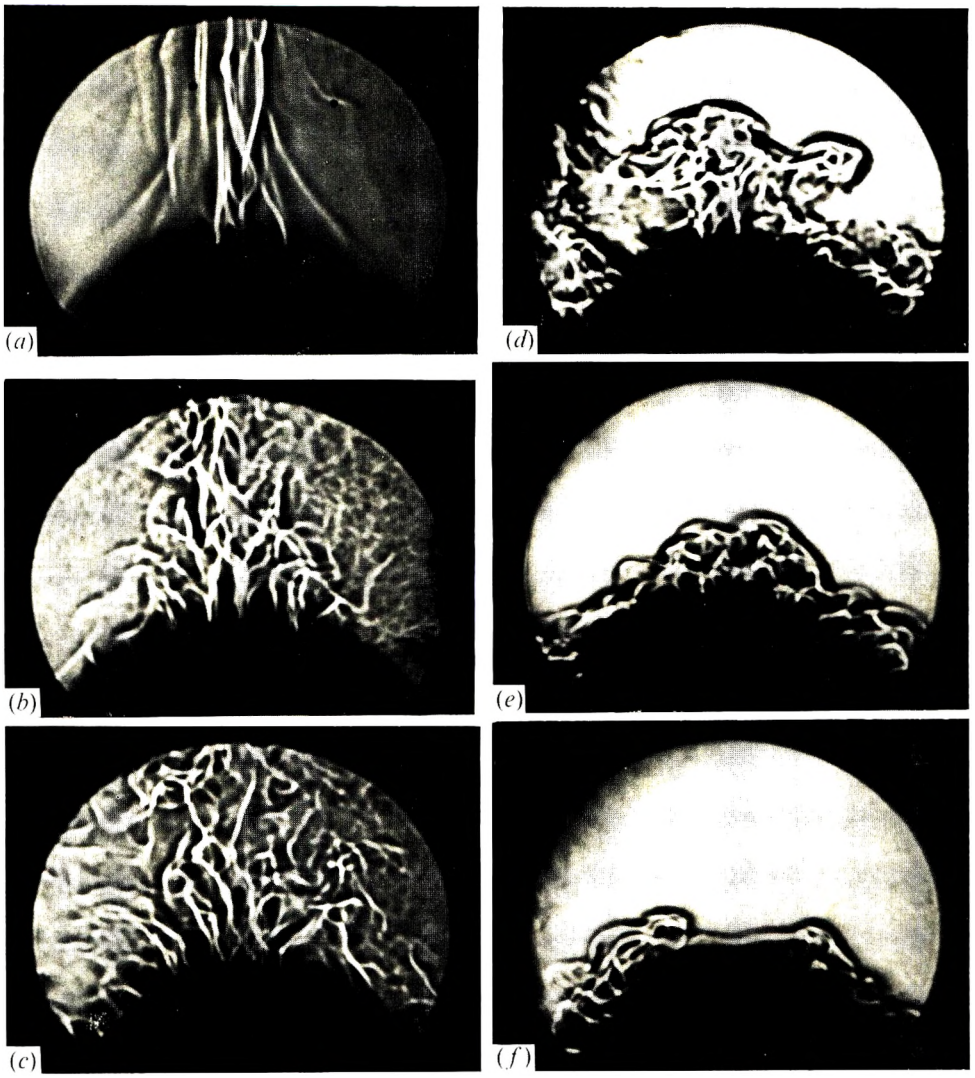

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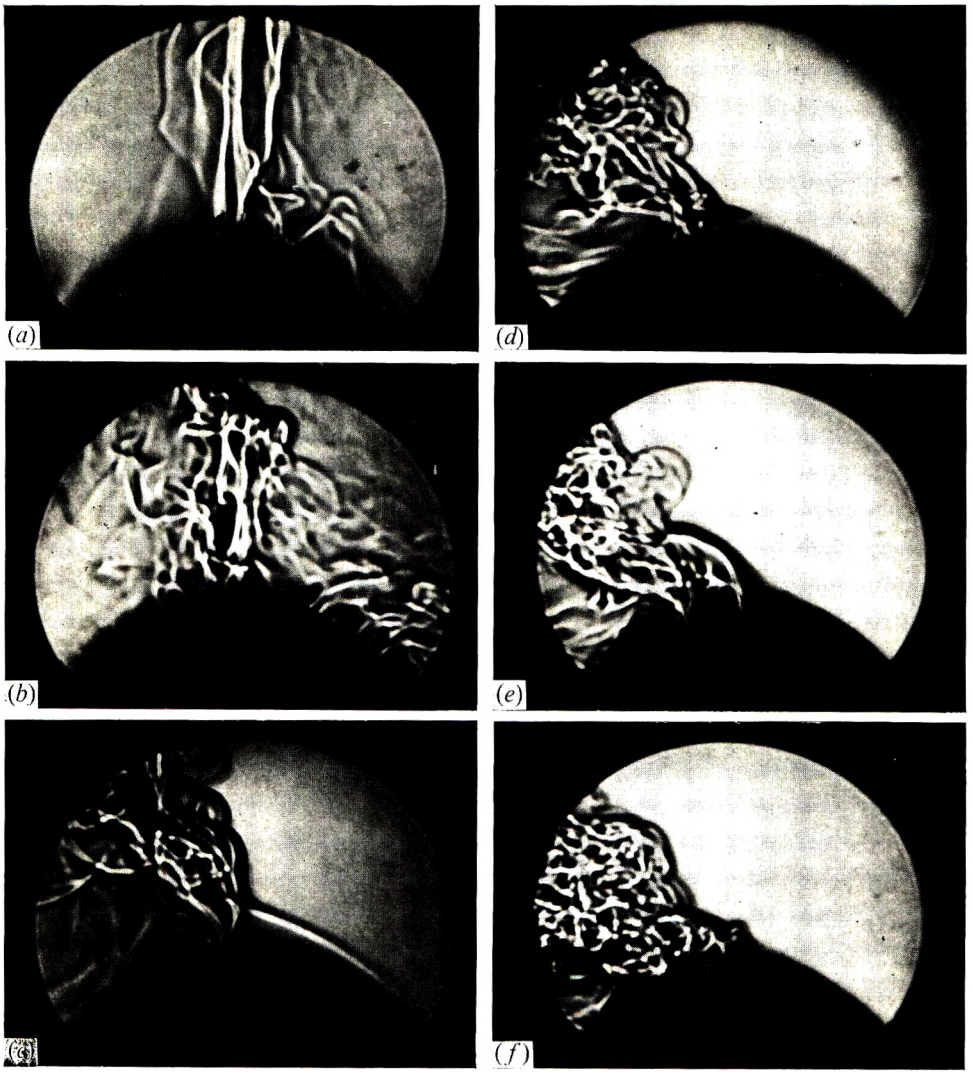
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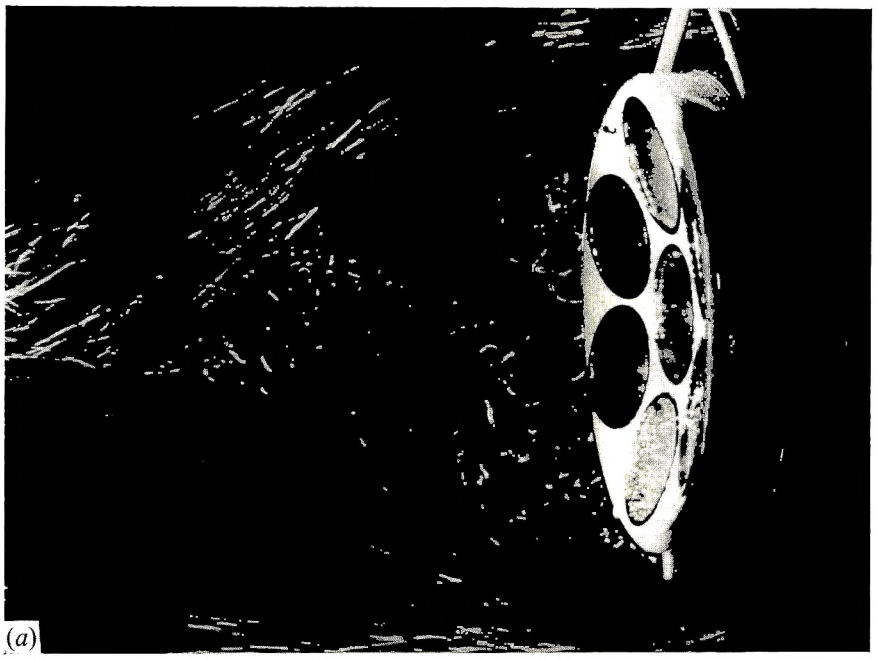

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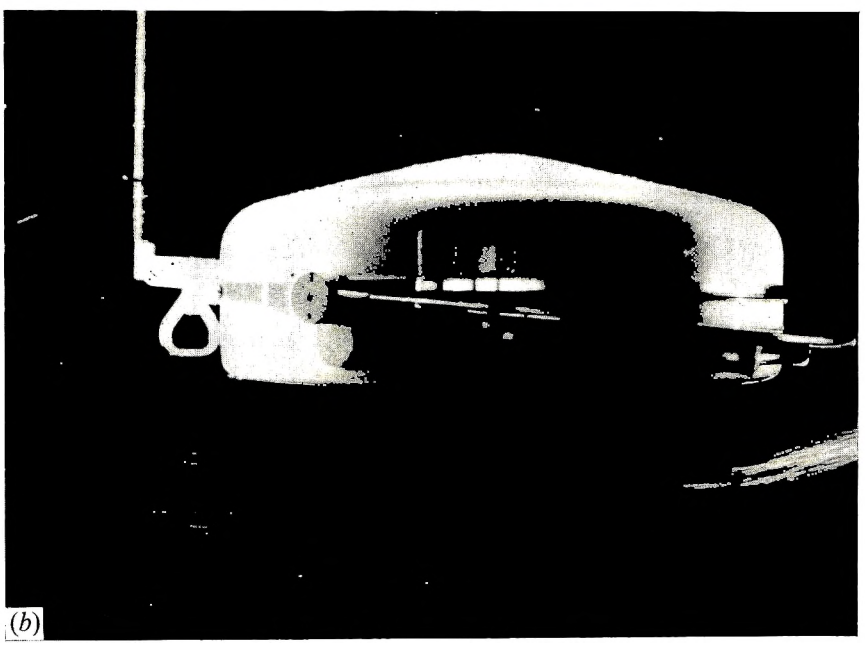

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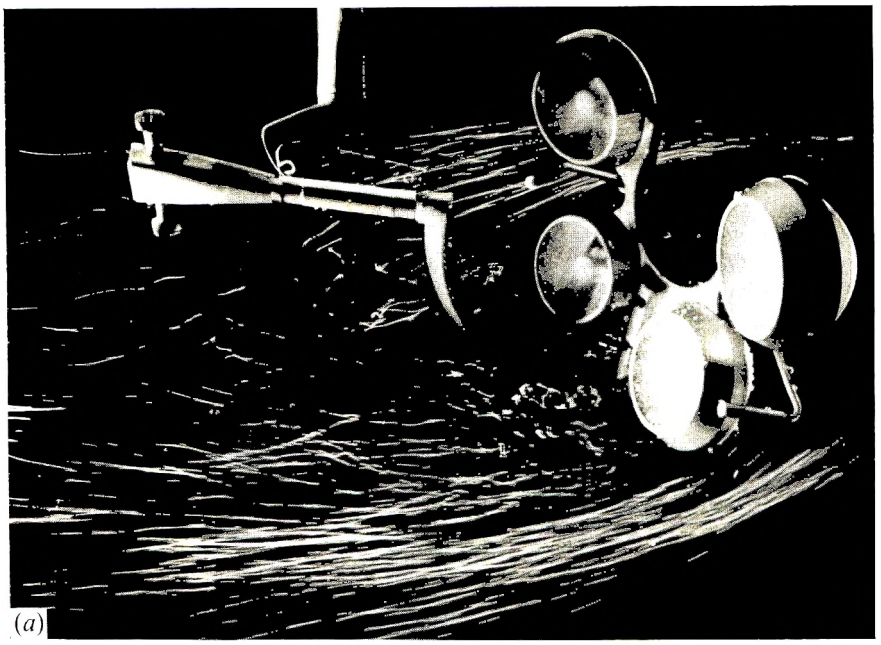

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EXPLANATION OF PLATES

PLATE 1

Effect of a downflow of air on the heat rising from a single pod of the Drayton Castle Daystar operating lamp. (*a*), 0 m./s.; (*b*) 0·1 m./s.; (*c*), 0·2 m./s.; (*d*), 0·3 m./s. (*e*), 0·4 m./s.; (*f*), 0·5 m./s.

PLATE 2

Effect of a crossflow of air on the heat rising from a single pod of the Drayton Castle Daystar operating lamp. (*a*), 0 m./s.; (*b*), 0·1 m./s.; (*c*), 0·2 m./s.; (*d*), 0·3 m./s.; (*e*), 0·4 m./s.; (*f*), 0·5 m./s.

PLATE 3

(*a*) Stagnant area resulting from the use of a conventional lamp in vertical laminar-flow conditions. Direction of flow from right to left.

(*b*) Airflow condition resulting from the use of a conventional lamp in horizontal laminar-flow conditions. Direction of flow from right to left.

PLATE 4

(*a*) Airflow resulting from the use of a modified Drayton Castle 'Daystar' lamp. Direction of flow from right to left.

(*b*) Turbulent effect from a person in a horizontal airflow of about 0·4 m./s.

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Viruses associated with acute respiratory infections 1961–71

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SUMMARY

Influenza-like illness, cold and sore throat was the diagnosis given in over 80% of 5177 acute respiratory illnesses in patients swabbed over a 10-year-period. A pathogenic organism was isolated twice as frequently from patients with a sore throat or an influenza-like illness as from those diagnosed as suffering from croup or laryngitis and bronchitis. A laboratory diagnosis was commoner in school children than in older or younger persons.

Most of the organisms isolated were found in association with all types of acute respiratory illness but, with increasing age of the patient, one particular agent or group of agents was more likely to be of aetiological significance.

INTRODUCTION

Over a 10 year period specimens were collected by general practitioners from patients under their care and submitted to the virus laboratory at Cirencester for isolation studies. The majority of these specimens were from patients thought to have an acute respiratory infection and in this paper the results of the virological examination of these illnesses have been used in an attempt to determine the relation between the nature of the illness and the infecting agent.

MATERIALS AND METHODS

The population studied, the type of specimens collected and the methods used to examine them have been described previously (Higgins, 1974).

RESULTS

Between October 1961 and September 1971 nose and throat swabs were received from 5177 acute respiratory infections of which 1243 (24·0%) were in pre-school children less than 5 years old, 1091 (21·1%) in school children between the ages of 5 and 15 years, 2837 (54·8%) in patients 16 years of age or older and in 6 (0·1%) the age of the patient was not recorded. Further sub-division of these three age groups was not possible because the number of specimens and organisms recovered from any one type of illness would have become too small for analysis. For similar reasons the agents isolated have been considered in groups rather than individual serotypes.

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Table 1. *Distribution of the patients sampled by age and clinical diagnosis*

Clinical illness	Age of patient				
	0–4 years	5–15 years	16 years and over	Not known	All ages
Cold	369 (29·7)	229 (21·0)	833 (29·4)	1 (16·7)	1432 (27·7)
Sore throat	126 (10·1)	370 (33·9)	548 (19·3)	—	1044 (20·2)
Croup or laryngitis	58 (4·7)	20 (1·8)	88 (3·1)	—	166 (3·2)
Tracheitis	93 (7·5)	70 (6·4)	145 (5·1)	—	308 (5·9)
Bronchitis	47 (3·8)	21 (1·9)	112 (3·9)	—	180 (3·5)
Bronchiolitis	150 (12·1)	12 (1·1)	11 (0·4)	—	173 (3·3)
Pneumonia	7 (0·6)	6 (0·6)	34 (1·2)	—	47 (0·9)
Influenza	391 (31·4)	356 (32·6)	1053 (37·1)	5 (83·3)	1805 (34·9)
Not known	2 (0·2)	7 (0·6)	13 (0·5)	—	22 (0·4)
All illnesses	1243	1091	2837	6	5177

Figures in parentheses = percentage of total sample from age group.

Influenza-like illnesses, colds and sore throats accounted for 83% of the illnesses sampled whereas tracheitis, bronchitis, bronchiolitis and croup or laryngitis each represented between 3 and 6% of the total illnesses examined virologically. Pneumonia was the only type of illness of which less than 100 cases were examined.

The proportion of the total number of specimens taken from each of the three age groups from patients with any one of the eight clinical diagnoses is shown in Table 1. Cases of bronchiolitis formed a much greater portion of the total samples from pre-school children than from school children or adults. Patients with a sore throat constituted twice and three times as large a proportion of the samples from adults and school children respectively than that from pre-school children. The commonest clinical condition swabbed in children of school age was a sore throat and such cases formed a third of the sample obtained from patients in this age group, whereas cases of croup or laryngitis, bronchitis and common cold figured less prominently than in the other two age groups. In older persons influenza-like illnesses and pneumonia formed a slightly larger proportion of the total sample than in younger persons.

Laboratory diagnosis

The relation between the ability to make a laboratory diagnosis and the clinical diagnosis is summarized in Table 2. In this respect illnesses fell into one of four groups. A diagnosis by the isolation of a human pathogen was made in over 40% of cases of sore throat and influenza-like illness, over 35% of cases of tracheitis and bronchiolitis, over 30% of cases of pneumonia and common cold but in only 20% of cases of croup or laryngitis and bronchitis. A laboratory diagnosis was more common in school children (43·3%) than in either pre-school children (36·5%) or adults (34·1%). The greater success with material from school children was the result of the relatively high isolation rates in this age group, from cases of sore throat, tracheitis and influenza-like illness – illnesses which represent almost three-quarters of the sample taken from children between the ages of 5 and 15 years.

An infecting agent was more frequently demonstrated in cases of croup or

Table 2. *Isolation rates by clinical diagnosis and age of patient*

Clinical illness	Age of patient				
	0–4 years	5–15 years	16 years and over	Not known	All ages
Cold	113/369 (30·6)	67/229 (29·3)	252/833 (30·3)	0/1	432/1432 (30·2)
Sore throat	55/126 (43·7)	200/370 (54·1)	193/548 (35·2)	—	448/1044 (42·9)
Croup or laryngitis	19/58 (32·8)	2/20 (10·0)	17/88 (19·3)	—	38/166 (22·9)
Tracheitis	31/93 (33·3)	30/70 (42·9)	47/154 (32·4)	—	108/308 (35·1)
Bronchitis	12/47 (25·5)	4/21 (19·0)	21/112 (18·8)	—	37/180 (20·6)
Bronchiolitis	55/150 (36·7)	3/12 (25·0)	3/11 (27·3)	—	61/173 (35·3)
Pneumonia	1/7 (14·3)	2/6 (33·3)	12/34 (35·3)	—	15/47 (31·9)
Influenza	164/391 (41·9)	164/356 (46·1)	420/1053 (39·9)	0/5	748/1805 (41·4)
Not known	1/2 (50·0)	1/7 (14·3)	2/13 (15·4)	—	4/22 (18·2)
All illnesses	451/1243 (36·5)	473/1091 (43·4)	967/2837 (34·1)	0/6	1891/5177 (36·5)

Numerator = number of isolations. Denominator = number of specimens examined. Figures in parentheses = percentage of specimens yielding a pathogen.

laryngitis in the 0 to 4 year old group than older persons as was seen also with bronchiolitis. However, the significance of the latter finding, like the low isolation rate in pneumonia in pre-school children, remains doubtful because of the small number of specimens received from these illnesses in at least two of the age groups.

Viruses associated with different clinical diagnoses

The relation between the clinical diagnosis and the infecting organism is shown in Fig. 1.

Colds. Rhinoviruses were the commonest agent found in association with the common cold and they were isolated from over 10% of specimens from children and 20% of specimens from adults with this clinical illness. In school children myxoviruses, and in all children under 16 years of age the paramyxoviruses, were isolated from approximately 5% of the specimens received.

Sore throats. Streptococci were the most frequently encountered organism in cases of sore throat except in pre-school children where they were second in importance to the enteroviruses. Enteroviruses, adenoviruses and herpes simplex virus were all found in association with this clinical condition but with reduced frequency as the age of the patient increased. In addition, paramyxoviruses in pre-school children, myxoviruses in school children and rhinoviruses in adults, made a significant contribution to the aetiology of sore throats.

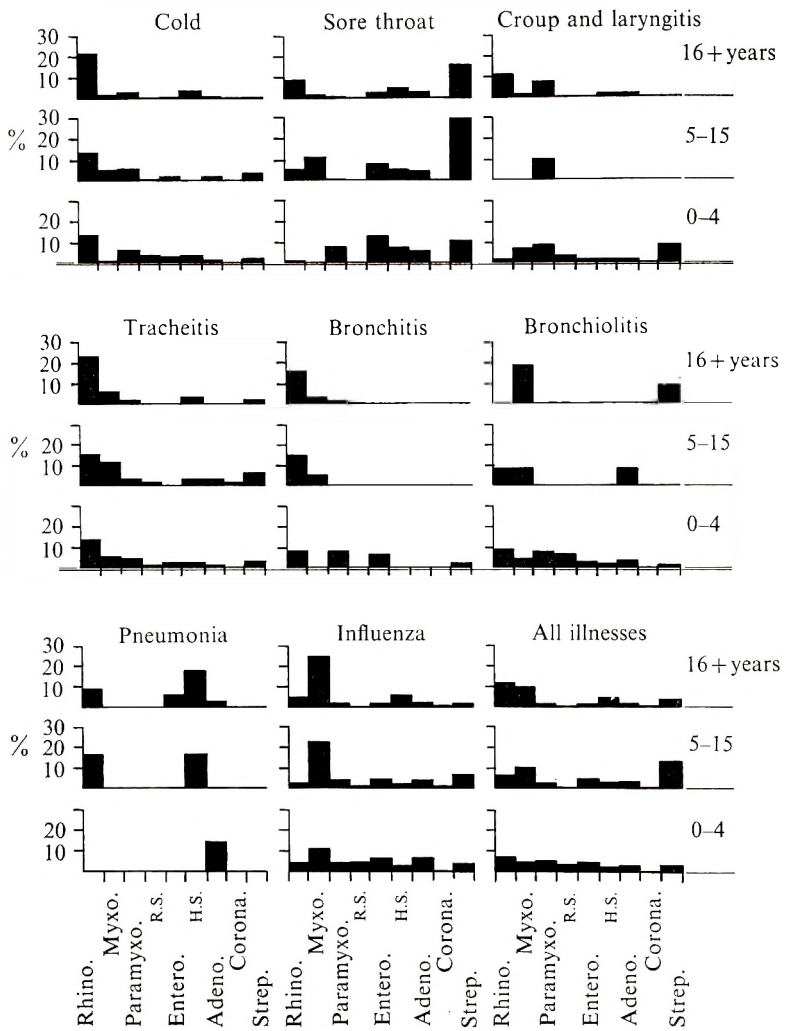

Fig. 1. Relation between clinical illness, age of patient and infecting agent. Rhino, Rhinoviruses; Myxo, Myxoviruses; Paramyxo, Paramyxoviruses; R.S., Respiratory syncytial virus H.S., Herpes simplex virus; Adeno, Adenoviruses; Corona, Coronaviruses; Strep, Streptococci groups A, C or G.

Croup or laryngitis. In school children paramyxoviruses were the only diagnosed cause of these conditions and with the rhinoviruses accounted for most of the proved infections in adults. Pre-school children with croup yielded a wider range of organisms with myxoviruses, paramyxoviruses and streptococci as the three most important.

Tracheitis. In all age groups rhinoviruses were the commonest agents isolated from cases of tracheitis and with a slightly higher frequency than from patients suffering from a cold. Myxoviruses were also of importance in all age groups but especially school children, and other agents were found in association with a small number of cases, notably in children.

Bronchitis. As with colds and tracheitis, rhinoviruses were the commonest

organism associated with bronchitis. Myxoviruses in school children and paramyxoviruses and enteroviruses in pre-school children were responsible for a smaller number of illnesses.

Bronchiolitis. Only three isolations were made from patients in each of the two older age groups and any conclusions drawn from these findings may be misleading. Of the 55 isolations from pre-school children with illnesses given this diagnosis, rhinoviruses, paramyxoviruses and respiratory syncytial virus were the commonest agents isolated but other viruses were also associated with this clinical illness in a smaller proportion of cases.

Pneumonia. Only in adults were sufficient agents isolated from patients with pneumonia to allow comment to be made. The commonest virus encountered was herpes simplex virus and, of the other agents, only rhinoviruses and enteroviruses were isolated from more than 5 % of cases examined.

Influenza-like illnesses. By far the commonest agent isolated, at all ages, from cases of influenza was a myxovirus. In adults herpes simplex virus and in school children streptococci were the only other agents isolated from more than 5 % of the specimens. In young children less than 5 years old, most viruses played a significant role in the causation of influenza-like illnesses although myxoviruses were the most important.

All respiratory illnesses. Respiratory illnesses in pre-school children most commonly yielded a rhinovirus, and coronaviruses were seldom found. All the remaining agents were isolated from between 3 and 6 % of specimens. Streptococci of groups A, C, or G and myxoviruses were each found in more than 10 % of specimens from school children whereas only rhinoviruses of the other agents were encountered in more than 5 % of specimens. Rhinoviruses and myxoviruses were each isolated from more than 10 % of specimens from adults but herpes simplex, the next commonest agent, was recovered from less than 5 % of patients.

Clinical diagnosis in cases of proved infection with different agents

Of the 5177 specimens from acute respiratory infections 1891 (36·5 %) yielded a virus or streptococcus belonging to groups A, C or G. The proportion of the total number of strains of any one group of viruses or streptococci associated with each of the eight clinical diagnoses is shown in Fig. 2. With the possible exception of respiratory syncytial virus, of which only seven strains were isolated from patients over the age of 5 years, the differences in these proportions between the three age groups were small and the results have been analysed for patients of all ages combined.

Approximately half (252) of the 496 rhinoviruses isolated were recovered from patients with a cold and a further 30 % were found in specimens from patients with a sore throat, tracheitis or an influenza-like illness. Over 80 % of the 462 myxoviruses isolated were from patients with influenza and no other form of illness yielded more than 5 % of the total number of strains isolated. Infection with one of the 148 paramyxoviruses isolated most commonly resulted in a cold (38 % of strains) and of the other diagnoses only influenza-like illnesses (29 % of strains) accounted for more than 10 % of the total number of viruses of this group that were

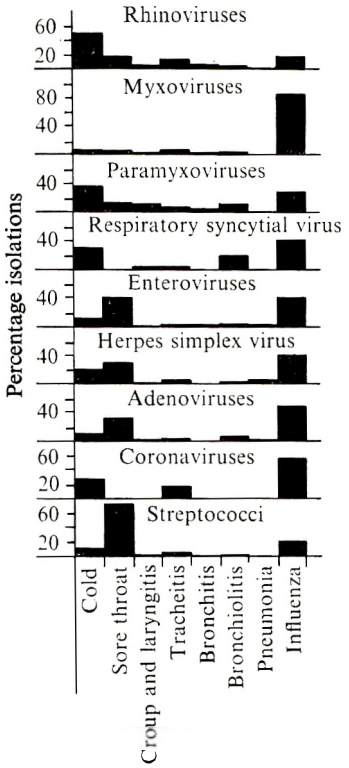

Fig. 2. Distribution of proved infections by diagnosis.

detected. Forty-nine strains of respiratory syncytial virus were isolated, most frequently (41 %) from cases of influenza-like illnesses but the common cold (31 %) and bronchiolitis (20 %) were also common sources of this virus.

Enterovirus isolations totalled 138, mainly from cases of influenza-like illness and sore throat, 40 % each, and 11 % of strains were isolated from cases of the common cold.

Herpes simplex virus, 181 strains, was most commonly associated with influenza-like illnesses (40 %) but 29 % of strains were isolated from cases of sore throat and 20 % from those with colds.

Adenoviruses were isolated on 119 occasions. Most strains were from patients with influenza-like illness (48 %) but 31 % were found in patients with a sore throat and 10 % in those with a cold.

Coronaviruses were isolated on 7 occasions only, from patients with an influenza-like illness, tracheitis or a common cold.

Streptococci of groups A, C or G – 291 strains were found, over 70 % of which were associated with a sore throat. Influenza-like illnesses yielded more than half of the remaining strains detected.

DISCUSSION

The lack of standardization of the clinical diagnoses among the various practitioners submitting specimens, and the absence of a random sampling system were two shortcomings of this study. Thus two identical illnesses could receive different diagnoses, and a biased selection of cases could invalidate the apparent frequency with which certain clinical illnesses resulted from infection with a particular group of viruses. Furthermore, differences in the ease with which different infecting agents can be isolated will affect the apparent importance of each group of agents.

If these limitations are accepted it will be realized that the observations made in this study are only an indication of the relation between the various organisms isolated and the clinical illness diagnosed. However, because the subjects studied were members of the general community the results are more likely to be related to the natural history of the infecting agents than those from investigations which are biased by the use of a selected population such as children in hospital or students.

This study has shown that most of the agents isolated are associated with all types of acute respiratory illnesses. However, their relative importance as causal agents of illnesses given a specific diagnosis varies with the age of the patient. The aetiology of all acute respiratory infections in young children under 5 years of age is broadly based in terms of infecting agents but in adults there is commonly one predominant organism which accounts for between 50 and 70 % of the agents associated with a particular clinical diagnosis.

The range of clinical illness found to be associated with proved infections has confirmed that all the agents isolated could be found in patients whose illness had been given any one of the clinical diagnoses used. However some types of illness were observed much more frequently as the result of infection with one agent than with another, e.g. rhinovirus infections commonly gave rise to colds, and myxovirus infections to influenza-like illnesses.

Coronaviruses were sought and isolated less often than any other group of viruses but the clinical illness with which they were associated was not restricted to an upper respiratory infection as has commonly been found previously (Hamre & Procknow, 1966; Bradburne, Bynoe & Tyrrell, 1967; McIntosh *et al.* 1967; Kapikian *et al.* 1969).

Although the specimens in this study were examined more extensively than in many earlier surveys, a diagnosis by isolation was made in only 36·5 % of cases studied. Until the examination of material from acute respiratory infections is more profitable it will not be possible to be certain how accurately this type of study reflects the true relation between clinical illness and virus infection.

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Antibodies against adeno-, cytomegalo- and rubella viruses in Australia-antigen-negative sera from patients with infectious hepatitis

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SUMMARY

Antibodies neutralizing adenovirus type 5 were found in all of 50 pairs (100 %) of sera from patients with acute icteric infectious hepatitis. The incidence in sera from the general population was 57 %. No differences in mean titre or in proportion of positive sera were found in the same sera tested for complement-fixing antibodies to cytomegalovirus and for antibodies to rubella virus haemagglutinin. The results can be interpreted as supporting the involvement, either direct or indirect, of adenovirus in the aetiology of infectious hepatitis; but could also be due to a non-specific anamnestic enhancement of the production of antibody to adenovirus, or to coincidental infection with adenovirus and the agent of infectious hepatitis.

INTRODUCTION

There have been a number of reports of raised antibody titres to a variety of bacterial and viral antigens in the sera of patients with liver disease (Bjørneboe, Prytz & Ørskov, 1972; Triger, Kurtz, MacCallum & Wright, 1972; Triger, Alp & Wright, 1972). Alwen (1968) reported that in a small group of sera from patients with acute icteric infectious hepatitis the proportion containing antibody neutralizing adenovirus type 5 was higher than in a group of normal control sera. No such increase was found in sera from patients with jaundice caused by infection due to *Leptospira* spp. Adenovirus has long been popular as one of the so-called 'candidate' viruses in infectious hepatitis (Hersey & Shaw, 1968). It was therefore of interest to extend the earlier work to a much larger group of sera from patients with acute icteric infectious hepatitis; and when possible to test sera from the same patients 3 months after the illness. Two other common viruses, cytomegalo virus and rubella were included in the survey.

MATERIALS AND METHODS

Sera

Sera from patients in the acute icteric phase of infectious hepatitis were obtained from Public Health Laboratory Service laboratories and from general practitioners throughout the country in response to a request published in the Communicable Disease Report through the courtesy of Sir James Howie of the Public Health Laboratory Service. Sera were also obtained from the same patients 3 months later. Specimens consisted of separated serum or whole blood without additives. The serum was removed from clotted blood and centrifuged at 1000 *g* for 10 min. to remove blood cells.

Through the courtesy of Dr S. J. Starkie, of St Mary's Hospital Medical School, London, W.2, control sera were obtained from a large collection obtained for tissue-typing from the siblings and relatives of patients with kidney disease, and from members of the general public. No medical histories were available for the donors of the control sera: but none of the donors were acutely ill when the specimens were taken.

Sera were stored frozen at $-70°$ C in sealed glass ampoules and inactivated at 56° C for 30 min. before use.

The mean age of the 27 men and 23 women from whom infectious hepatitis sera were obtained was 20 yr.; and of the 25 men and 25 women in the control group, 22·6 yr.

Adenovirus neutralization test

The adenovirus type 5 used throughout the neutralization tests came from the same batch grown in HeLa cells and stored at $-70°$ C. The virus was originally obtained from Dr H. G. Pereira of the National Institute for Medical Research, London, N.W.7. HeLa cells were originally obtained from Flow Laboratories Ltd. The cells were cultured in Eagle's minimum essential medium; subcultured twice a week, and always used for neutralization tests 2 days after subculture. A rapid neutralization test was used in which cultures of HeLa cells were inoculated with mixtures of adenovirus type 5 and dilutions of the test serum. Cytopathic effect caused by unneutralized adenovirus was assessed 2 days later by counting cells microscopically. The neutralization titre was calculated and expressed as the reciprocal of the dilution of serum that reduced by 50 % the proportion of cells showing characteristic nuclear cytopathic effect (Boyer, Denny, Miller & Ginsberg, 1960) in control cultures inoculated with fetal bovine serum in place of the test serum. Titres of 1/5 or greater were considered positive. Sera were coded before testing: 2 sera from patients in the acute phase of hepatitis, sera from the same patients 3 months later, and 2 control sera were tested at one time.

Complement-fixation test for cytomegalovirus

Antibodies to cytomegalovirus were measured in complement-fixation tests by a technique similar to that of Bradstreet & Taylor (1962). The tests were done in disposable micro-titre trays (Flow Laboratories Ltd). The antigen and positive reference serum were obtained from Dr C. M. Patricia Bradstreet, Standards

Laboratory, Central Public Health Laboratories, Colindale, London, N.W.9. The complement, sheep erythrocytes and haemolytic serum were obtained from Burroughs Wellcome & Co., Beckenham, Kent. Paired sera were tested in parallel. Titres of 1/2 or greater were considered positive.

Haemagglutination-inhibition test for rubella virus

Antibodies to rubella haemagglutinin were measured by a micromethod (Stewart *et al.* 1967). The lyophilized antigen was obtained from Flow Laboratories Ltd. Non-specific haemagglutination inhibitors were removed from test and control sera with manganous chloride and heparin (Feldman, 1968). Antigen controls and known positive and negative serum controls were included in each group of tests. The end-point was taken as the reciprocal of the highest dilution of serum showing complete inhibition of agglutination. Titres of 1/4 or greater were considered positive.

Australia antigen

All sera were tested for Australia antigen and antibody by immunodiffusion (W.H.O., 1970) using an Australia antigen detection kit supplied by Behringwerke AG. Two of the sera from patients with infectious hepatitis were positive for Australia antigen, one for antibody, and one for both; they were discarded from the series. None of the control sera were positive for either Australia antigen or antibody.

RESULTS

The distribution of the titres of the sera from patients with acute infectious hepatitis, of sera from the same patients three months later, and of the controls, is shown in Fig. 1. All patients with hepatitis had antibody that neutralized adenovirus in their sera. Two of the acute phase sera had titres of less than 1/5; but 3 months later the serum from each of these patients contained antibody. One convalescent serum had a titre of less than 1/5 though serum from the same patient during the acute phase of the illness was positive.

Antibody that neutralized adenovirus was found in only 57 % of the sera in the control group.

The geometric mean titres were 1/84 for the acute phase sera, 1/81 for the convalescent phase sera and 1/14 for the control sera. The mean titres of the acute phase and control sera, omitting negative results, were 1/103 and 1/93 respectively, and did not differ significantly by Student's t-test. The mean titres of the acute phase sera from patients with hepatitis and the control group were 1/9 and 1/11 respectively, and did not differ significantly. Two of the convalescent sera showed a twofold and 3 a fourfold rise in titre; and 2 of the convalescent sera showed a twofold and 1 a fourfold drop in titre.

In the haemagglutination test for rubella virus, the proportion of positive sera (titres > 1/4) in patients with hepatitis was 82 %; and 86 % in the control group. The mean titres for the acute, convalescent and control sera were 1/52, 1/52 and 1/78 respectively. The mean titres of the hepatitis sera and the control sera were

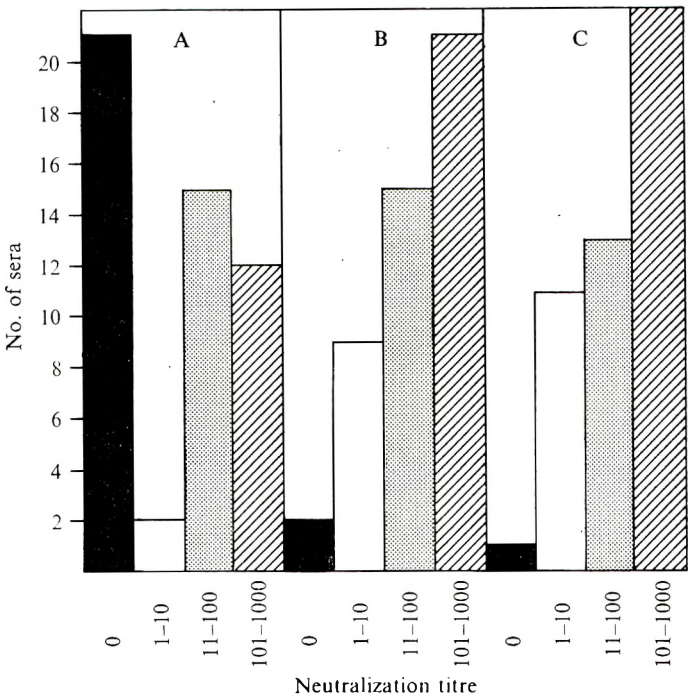

Fig. 1. Distribution of titres of antibody neutralizing adenovirus type 5. A, control sera; B, acute phase sera; C, convalescent phase sera.

not significantly different. Four convalescent sera showed twofold increases and 2 fourfold increases in titre; and 8 of the convalescent sera from patients with hepatitis showed a twofold drop and 6 a fourfold drop in titre.

DISCUSSION

These results demonstrate an increase in the proportion of sera from patients with acute icteric infectious hepatitis that are positive for antibody neutralizing adenovirus type 5. Antibody was detected in all of 50 pairs (100 %) of infectious hepatitis sera; but in only 28 of 50 control sera (57 %). The latter figure agrees well with published estimates of the prevalence of antibody to adenovirus type 5. For example Huebner *et al.* (1954) reported 53 % for a large group of sera from the general population between the ages of 16 and 34 years. However, Potter, Shedden & Zachary (1963) reported only 20 % in a survey of the general population. Twelve sera in our test group showed a twofold or greater rise in titre compared with 5 that showed a twofold or greater drop. However, in 76 % of paired sera there was little or no difference in titre between the first and second specimens.

Although it cannot be excluded, the possibility of a direct involvement of adenovirus type 5 in the aetiology of infectious hepatitis, – as for example, in canine infectious hepatitis in which the liver damage is caused by the multiplication of an adenovirus – appears unlikely despite the various isolations of adenoviruses from cases of the disease (Davis, 1961; Hatch & Siem, 1966; Hartwell, Love & Eidenbock, 1966). An indirect action of adenovirus is perhaps more

probable; this could arise from adenovirus multiplication elsewhere than in the liver, with consequent damage by adenovirus antigen(s), or antigen(s)/antibody complexes.

Non-specific heterologous enhancement was first described by Weil & Felix (1916), who described a non-specific enhancement of antibodies to *Salmonella typhi* during infection with typhus. Our results could be interpreted as heterologous enhancement of antibody against adenovirus by acute infectious hepatitis without involvement of adenovirus in pathogenesis. The icteric phase of acute infectious hepatitis, though the major clinical symptom, may be a sequel to the main phase of the disease, so that antibody stimulation might have preceded the time at which our first samples were taken, possibly by up to 3 weeks. This could explain why in 76 % of the hepatitis sera the titres of antibody neutralizing adenovirus were similar in the acute and convalescent phases of the disease. It would be of great interest to measure the neutralizing antibody levels in a group of pre-icteric sera. After stimulation it would be expected that the adenovirus-neutralizing antibody titres would remain raised for some time (Sohier, Chardonnet & Prunieras, 1965).

Nuromskaya, Potulova & Fedenko (1968) suggest that patients with infectious hepatitis are predisposed to simultaneous infection with an adenovirus. Closs (1972) has also reported a rather unusual association between an outbreak of acute infectious hepatitis and rubella virus in which the two diseases caused simultaneous outbreaks with exactly similar incubation periods. The high proportion of sera from infectious hepatitis patients positive for adenovirus neutralizing antibody may be due to simultaneous infection with the agent of infectious hepatitis and adenovirus. Infectious hepatitis frequently begins with a sore throat (Glover & Wilson, 1931) and lymphadenopathy (Finks & Blumberg, 1945), which could be due to infection with adenovirus rather than to the multiplication of the agent of infectious hepatitis.

The possibility that the high titres of antibody neutralizing adenovirus type 5 are due to impairment of the normal mechanism for removing continuously released antigens with consequent enhancement of antibody production, is of particular interest in relation to recent reports concerning raised antibody titres to a number of unrelated antigens in infectious hepatitis. Triger, Kurtz *et al.* (1972) demonstrated raised titres to measles and rubella viruses, though not to coxsackie B1 and B5, *Herpes simplex*, parainfluenza type 1, or *Mycoplasma pneumoniae*, in chronic active hepatitis. Bjørneboe *et al.* (1972) reported an increased frequency of agglutination reactions with 10 different antigens of *Escherichia coli* group O in the serum of patients with cirrhosis of the liver; and Triger, Alp *et al.* (1972) described an increase in antibody titre to *Escherichia coli* and *Bacteroides* in patients with liver disease. Patients with acute viral hepatitis were included in the group and showed increased incidence of antibody to *E. coli* and *Bacteroides*; but not to *Haemophilus influenzae* B which is not commonly found in the gastrointestinal tract. Protell *et al.* (1971) demonstrated an increased proportion of sera positive for agglutinins to *Salmonella* antigens in patients with chronic active liver disease. It therefore appears that antibodies to a number of antigens present in the

gastrointestinal tract in health are increased in titre, in incidence, or in both, during certain diseases of the liver including acute viral hepatitis. Both Bjørneboe (1971) and Triger, Kurtz *et al.* (1972) suggest that in some diseases of the liver its ability to sequester antigens is reduced; or that antigen already sequestered and rendered temporarily non-antigenic is released and becomes able to stimulate antibody production; and that such increases may largely account for the hyperglobulinaemia common in liver disease. In support of this hypothesis are the observations that after the establishment of a portacaval shunt in cirrhosis the level of immunoglobulins is greater than in cirrhotics without a shunt, and that the incidence of agglutinins to intestinal microbes is highest (Bjørneboe *et al.* 1972).

The hypothesis could also explain the increased proportion of sera positive for antibody neutralizing adenovirus in acute viral hepatitis, as adenovirus infections may persist in an occult form associated with low levels of circulating antigen and antibody.

Most of the micro-organisms to which antibodies are increased in liver diseases are commonly found in the gastrointestinal tract. Although adenovirus infections are most commonly encountered in the upper respiratory tract they can also affect the gastrointestinal tract, which is the usual route of vaccination against them. Neither rubella nor cytomegalovirus commonly affect the gastrointestinal tract. In this survey the proportion of hepatitis sera positive for antibody to rubella haemagglutinin and cytomegalovirus was no higher than in the control group.

Titres of antibodies fixing complement with adenovirus type 5 were not measured in this survey. Hatch & Swanson (1969) investigated the incidence of complement-fixing antibodies to adenovirus type 4 and to the San Carlos agent No. 8 (an atypical adenovirus type 3, isolated from a case of infectious hepatitis). The incidence varied from 61 to 67 % in acute and convalescent hepatitis sera, and from 52 to 56 % in normal controls. The titres in the two groups were not significantly different. Alwen (1968) also found no increase in complement-fixing antibodies to adenovirus type 5 in a small group of sera from patients with acute viral infectious hepatitis; though the proportion of sera positive for neutralizing antibody was increased.

We think it probable that the increased proportion of sera positive for antibody neutralizing adenovirus type 5 from patients with infectious hepatitis is the result either of a heterologous immunological enhancement, or a specific stimulation of the production of antibody facilitated by impairment of the process by which continuously released antigen from a chronic infection is sequestered. Although sera from patients with acute icteric Weil's disease due to *Leptospira* spp. do not show increased titres of antibody neutralizing adenovirus (Alwen & Fulton, 1968), and increases in antibody to adenovirus may not be a general feature of diseases of the liver causing jaundice, it would be of great interest to extend the survey of antibody neutralizing adenovirus to include sera from patients with liver diseases other than infectious hepatitis, and particularly to sera from patients with Australia antigen.

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An outbreak of cholera in Australia due to food served in flight on an international aircraft

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SUMMARY

An outbreak of cholera occurred in November 1972 among passengers on an aircraft that had flown from London to Sydney. The infection was confined to economy-class passengers and the available evidence indicates that it was due to a dish of hors d'œuvres served on the aircraft between Bahrain and Singapore. Although one person died, the infection was generally mild, and almost half of those infected were symptomless. There was a significant difference between the immunization status of persons with clinical illness and the immunization status of other passengers. Current cholera immunization appeared to play a significant role in preventing symptoms of the disease, but it did not prevent a person becoming a carrier of the organism.

INTRODUCTION

Because modern air travel freely permits rapid movement from one country to another, it has played an important part in the widespread geographical distribution of cholera during the present pandemic. Cholera has been introduced into Australia, France, Ghana, West Germany, Japan, Sweden, Taiwan, and the United Kingdom by this means (Barua, 1972). However, no report has yet been published of an incident in which a large number of passengers have become simultaneously infected with *Vibrio cholerae* as a result of consuming food or water on board an aircraft. The following report describes such an event. At least 47 persons were infected, and cholera introduced into three different countries as a result of the serving of contaminated food to passengers on a Boeing 747 flight from London to Sydney.

THE OUTBREAK

On 5 November 1972, a patient, who had just arrived by air from overseas, was admitted to a hospital in Sydney, N.S.W., with severe watery diarrhoea. Faeces were collected for culture, and by the following day a presumptive diagnosis of cholera had been made. Further laboratory tests classified the organism as *Vibrio cholerae* El Tor, serotype Inaba. By 7 November two other persons, who had been passengers on the same flight to Sydney, had also been admitted to hospital with a presumptive diagnosis of cholera. As it seemed likely that this outbreak of

cholera might involve others who had travelled on the same aircraft, the Australian Department of Health and the several State Health Departments immediately set about contacting all passengers and crew. By 11 November this had been achieved and arrangements made for the collection and examination of faecal specimens. Persons giving positive laboratory tests for cholera and any suffering from diarrhoea were placed in quarantine. The remainder were put under surveillance. Although there were a few exceptions, in general no attempt was made to immunize the passengers, their contacts or members of the public.

Numbers involved

The aircraft, a Boeing 747, had flown from London to Sydney via Amsterdam, Bahrain, Kuala Lumpur and Singapore. It had an almost complete complement of passengers – 26 first-class and 331 economy-class – when it left Bahrain. Of these, 40 disembarked at Singapore. Two of these disembarking passengers, who later continued their journey to Australia on another flight, were subsequently shown to have positive tests for *V. cholerae*. Forty-seven passengers joined the aircraft in Singapore.

A total of 47 passengers, including 6 persons who had continued their journey to New Zealand on another aircraft, were found to be excreting *V. cholerae* with or without overt signs of illness. No passengers who first boarded the aircraft in Singapore, no crew member, and no passenger travelling first-class, was found to have a positive test for cholera. Only economy-class passengers were subsequently shown to be infected. In what follows the term 'case' will be used to describe a person with a positive laboratory test for *V. cholerae*, and with some (mild to severe) overt signs of illness. The term 'carrier' will be used to describe a person with a positive laboratory test for *V. cholerae*, but with no overt signs of illness. On this basis, of the 47 infected passengers, 25 would be classified as cases and the remaining 22 as carriers.

Although faecal samples were collected from all passengers and crew members, it is possible that some carriers were not detected, either because they were no longer excreting vibrios at the time of examination, or because the laboratory methods used were not sufficiently sensitive. The actual number of persons infected may therefore have been higher than the figures given here indicate.

EPIDEMIOLOGY

Probable source of the infection

Three possible ways in which the infection might have occurred were considered initially. The first was as a result of eating food or drinking water in an airport terminal *en route* to Australia, but it soon became apparent that many of those infected did not eat or drink in any terminal. This was, therefore, considered unlikely to be the source of the infection. The second possibility considered was that the infection occurred as the result of chance contamination of food by a carrier among the cabin staff on the aircraft. When it became known that a large number of persons had been infected this hypothesis seemed unlikely. It was also

learned that, during flight, the meals were stored as pre-packed individual servings on trays in refrigerated modules, and there was little handling of food by the cabin staff. The third and by exclusion the most likely explanation appeared to be that the source of the infection was food or water loaded on the aircraft at Singapore or Bahrain.

In addition to the infections that occurred on the London–Sydney flight, two cases of cholera were detected in England among passengers on a Boeing 747 flight from Sydney to London. This aircraft left Bahrain for London 2 hr. before the eastbound aircraft left Bahrain for Sydney. Singapore and Bahrain were the only stopover points common to both flights.

It has already been stated that all 47 persons infected on the London–Sydney flight were on the aircraft when it left Bahrain; none of the passengers who boarded the aircraft for the first time at Singapore were found to be infected. In addition, two passengers, who had been on board when the aircraft left Bahrain, but who disembarked at Singapore and travelled to Sydney 3 days later, were found to be excreting *V. cholerae*. One of these persons had suffered from diarrhoea while in Singapore.

At the time of the outbreak, Bahrain was experiencing an outbreak of cholera that had begun on 24 October 1972. The outbreak was due to *V. cholerae* El Tor, serotype Inaba, phage type 2, the same type responsible for the infection among the passengers on both aircrafts. This information was kindly provided by the W.H.O. Cholera Reference Laboratory, Calcutta who phage typed the strains of *V. cholerae* from Bahrain and from the cases on the aircraft. Singapore was considered to be 'cholera-free' at the time.

On the available evidence, therefore, it appeared most likely that water or food loaded on the aircraft at Bahrain was the source of the infection. For the purposes of this discussion, the possible roles of water and of food will be discussed separately.

Water

On the Boeing 747, domestic water is loaded on the aircraft through only one filling point. It is then, for engineering reasons such as the trim of the aircraft, fed into three separate tanks of approximately 100 gallons each. It passes from these tanks into one common manifold which services all parts of the aircraft. The water used in first-class and economy-class compartments is therefore the same, and the same water is also used by the 19 crew members, but no case or carrier of cholera was found among first-class passengers or crew. Furthermore, many of the victims were certain they did not drink water, eat ice, or even clean their teeth during the flight. In the economy-class, all drinking vessels are disposable and are not washed during flight. This would appear to be good evidence against the infection being due to contamination of the water supply on the aircraft.

The water used for filling the water carts which service aircraft at Bahrain is also used in the food catering unit and in the snack bars and other amenities at the airport terminal. If there had been a general contamination of this water supply, clinical cases of cholera would probably have occurred among passengers on other airlines and among staff at the airport complex. This was not the case.

Table 1. *Percentage of infected and non-infected passengers who ate the various items on the tray of hors d'œuvres*

Food item	Infected passengers (%)	Non-infected passengers (%)
Pâté	55	50
Duck	83	52
Smoked salmon	74	64
Salami	60	60
Mushroom salad	79	44
Stuffed egg	73	*

* Omitted inadvertently from the questionnaire sent to these passengers.

In addition, several other aircraft were serviced in Bahrain both before and after departure of the two aircraft on which cholera occurred. There were no cases of cholera among passengers on these other aircraft.

Therefore, although it is not possible to be absolutely certain, the available evidence indicates that water loaded on the aircraft at Bahrain was not the cause of the infection.

Food

The meals served to first-class and to economy-class passengers and to crew members were different. Two economy-class meals were loaded aboard at Bahrain. The first meal served was a breakfast consisting of grapefruit cocktail, cereal and milk, bacon, egg, mushrooms and grilled tomato, together with bread, butter and marmalade. It seems unlikely that this meal could have been the cause of the infection. The cereal and milk were eaten by only a small number of the cholera cases. The bacon, eggs, mushrooms and tomato were heated on board the aircraft and it is almost certain that the cooking would have killed any vibrios likely to be present. The grapefruit cocktail was prepared in Bahrain from fruit imported in tins. The pH of this product is within the range 3·5–4·5 which is unsuitable for the growth and survival of *V. cholerae*.

The second meal consisted of cold assorted hors d'oeuvres (pâté in aspic, smoked salmon, glacé duck, salami, stuffed egg and a mushroom salad), an apple flan (individually packed in cellophane wrapping), bread, butter, cheese and biscuits. Tea, coffee and milk were also available.

An almost identical meal was prepared for the westbound flight departing Bahrain for London 2 hr. before the eastbound flight left for Sydney. Items on the hors d'œuvres plate served on both aircraft were prepared by the same staff according to the same recipes from the same batches of food. The breakfast meal was not common to both flights. Cases of cholera were reported among passengers on both flights. These findings strongly support the theory that a hors d'œuvre item was the vehicle of infection.

It has proved impossible to determine which particular item was responsible for the infection. Indeed, because they were arranged very close together on a

Table 2. *Growth of* V. cholerae *El Tor at* 28° *C. in the various food items. 'Good' indicates an increase of at least* 3 *logarithms and 'Fair'* 1–2 *logarithms in the cholera count*

Food item	pH	Growth of *V. cholerae*
Duck	6·9	Good
Pâté	6·5	Fair
Aspic	4·7	Nil
Smoked salmon	5·4	Poor
Salami	5·1	Nil
Stuffed egg	7·15	Very good
Mushroom salad	5·3	Nil
Milk-cream	6·8	Very good

small tray, cross-contamination from one to another, either directly or by means of eating utensils, could readily have occurred. However, an attempt was made to discover which passengers ate which items – infected persons by direct questioning and the remaining passengers by means of a postal questionnaire. The results are summarized in Table 1, but they should be interpreted with caution. Many passengers, particularly older passengers, had difficulty in recalling which items they had indeed eaten. One passenger, a carrier, claimed not to have consumed either food or drink during the entire flight between Bahrain and Singapore. Although Table 1 would seem to show that proportionately more infected than non-infected passengers ate the various hors d'œuvres, and to that extent supports the view that these items were the vehicle for the infection, it provides no evidence concerning which item or items were infected.

Of the food items served as part of the cold meal only the chicken pâté, cold duck, stuffed egg and the 50–50 milk-cream were considered to be possible sources of the infection. The remaining food items were served on other aircraft or were later shown to be poor substrates for the growth of *V. cholerae* (Table 2).

Both the pâté and the duck were cooked the day before serving and stored overnight in a refrigerator. The pâté was covered in aspic and encased in a pastry crust. The same aspic, diluted with water, was used to glaze pieces of duck (breast meat) which were arranged, by hand, on 'squares' of toasted bread. To prepare the stuffed egg, hard-boiled eggs were cut in half, the yolks removed, mixed with cream and placed in a piping bag. This mixture was used to fill each egg white. The egg proved to be a very good substrate for the growth of *C. cholerae*. The milk-cream mixture was prepared away from the main kitchen at a commercial dairy in Bahrain. It was also used for crew meals, and is less likely to be the vehicle of infection, but cannot be completely eliminated, as it was taken by most passengers in tea or coffee and was also served with cornflakes for breakfast.

Meal preparation and assembly

The final preparation and assembly of the meals served on the London–Sydney flight began at 2.00 p.m. Bahrain time on 2 November, and preparation for the London bound flight began approximately 3 hr. later. Both sets of meals were placed in Boeing 747 food modules and stored in the refrigerated-module holding

Table 3. *Incubation period for the 25 cases of cholera among passengers on the London–Sydney flight*

Incubation period (hr.)	Number of cases
–24	1
24–48	16
49–72	6
72–	2

area from about 7.00 p.m. until they were loaded on the flights at 2.00 a.m. (westbound) and 4.00 a.m. (eastbound) on 3 November. Meals were eaten on both flights at approximately the same time, viz. 12.00 noon Bahrain time, 3 November, which was almost 24 hr. after preparation and assembly.

Refrigeration space in the flight kitchen at Bahrain was adequate but there is a possibility that the items prepared at 2.00 p.m. were not refrigerated until both meals were completed, i.e. 7.00 p.m. Growth of contaminating organisms in the foods could have taken place during this period.

Investigation of catering staff

At least three faecal specimens were collected from every member of the staffs of the airport catering unit, the terminal snack bar and the dairy – none of whom was known to have suffered from a cholera-like illness at or about the time in question. The specimens were examined in Bahrain for *V. cholerae* and two members of the catering unit were found to be excreting the organism, although one was positive only in one out of three samples. One of these two carriers had taken part in the preparation of the hors d'œuvre meal served on the aircraft. He was a butcher's assistant who helped chop and mince meat and poultry used in the aircraft meals. The two carriers were not detected until approximately 2 weeks after the outbreak occurred in Australia, and it cannot be stated definitely that either was the source of the infection. However, the findings do indicate that there is a definite possibility that a carrier may have been present in the kitchen at the time of the outbreak and contaminated one or more of the food items served on the aircraft.

CLINICAL FINDINGS

Symptoms (diarrhoea and, occasionally, vomiting) occurred in most cases 24–48 hr. after eating the suspected meal (Table 3). None of the passengers was ill on the aircraft. In general the severity of the illness was mild (Table 4). Thirty-two of the 47 persons infected were either symptomless or suffered only minimal symptoms of one or two loose stools. Of eight cases classified by the attending physicians as severe, five had rice-water stools and symptoms resembling classical cholera. One New Zealand patient, a male aged 65, died. The duration of the illness ranged from a few hours to several days.

Table 4. *Severity of symptoms of the* 47 *persons infected with* V. cholerae

Severity of symptoms	Number of persons
None (carrier)	22
Mild	10
Moderate	7
Severe	8

Table 5. *Comparison of the age distribution of cholera patients with that of all passengers on the London–Sydney flight*

Age group	Cases		Carriers		All patients		All passengers	
	No.	%	No.	%	No.	%	No.	%
–30	1	4	2	9	3	6	68	18
31–40	1	4	1	5	2	4	42	11
41–50	1	4	1	5	2	4	44	12
51–60	3	12	5	23	8	17	55	15
61–70	10	40	9	41	19	40	96	26
70–	9	36	4	18	13	28	68	18
All ages	25	—	22	—	47	—	373	—

Age distribution

The age of all passengers on the aircraft was obtained from disembarkation cards completed by each passenger (Table 5). From these it was found that 44 % (164/373) of all passengers who disembarked in Australia, 76 % (19/25) of the clinical cases and 59 % (13/22) of carriers were older than 60 years. The difference in age distribution of infected and non-infected persons is significant – the infection being more likely to occur in older persons. Seven of the eight passengers who were severely ill were aged 60 years or over.

Sex distribution

The sex of all passengers on the aircraft was similarly obtained from disembarkation cards; 39 % were male and 61 % female. Of the 47 persons known to be infected with *V. cholerae*, 16 (34 %) were male and 31 (66 %) were female. Therefore, there was no significant difference between the sexes in regard to their susceptibility to *V. cholerae* infection.

Cholera immunization status

Two hundred and twenty non-infected passengers were asked, either personally or by letter, for details of their cholera immunization status, including the date and place of their last inoculation. Two hundred and eleven persons replied to this request. Of these, 28 joined the aircraft in Singapore and, therefore, did not eat the suspected meal. All of these passengers were immunized against cholera, as the Australian quarantine regulations in force at that time required all persons entering Australia from Singapore to be immunized. The immunization status of the remaining 183 persons together with that of all passengers infected with *V. cholerae* is given in Table 6. There was a significant difference between the

Table 6. *Cholera immunization status of infected and non-infected passengers*

Immunization status	Infected passengers		Non-infected passengers
	Cases	Carriers	
Current	1 (4%)	7 (32%)	55 (30%)
Not current	24 (96%)	15 (68%)	128 (70%)

immunization status of persons with clinical illness, and that of other passengers. Current cholera immunization did appear to play a significant role in preventing symptoms of the disease, but it offered no protection against a person becoming a carrier of the organism.

BACTERIOLOGY

Bacteriology of food

Although it was not possible to submit samples of the food items actually served on the two flights to bacteriological examination, duplicate samples of the hors d'œuvres served on these flights were prepared in Bahrain some two weeks after the episode occurred and sent to the School of Public Health and Tropical Medicine, University of Sydney, for examination.

Microbiological analysis of the pâté, duck, aspic, smoked salmon, salami, egg and the mushroom salad was carried out using standard methods. No *V. cholerae* were detected and, although small numbers of faecal coli were found in the duck, pâté and egg, the microbiological quality of the foods was generally satisfactory.

A specimen of the water on board the aircraft collected after its arrival in Melbourne, Australia, was also examined. The plate count (at 30° C.) was $1{\cdot}1 \times 10^4$ organisms per ml, but no *E. coli*, coliform bacilli or *V. cholerae* were detected. However, the water loaded on the aircraft in Bahrain had by then been almost completely replaced by water taken on at Singapore and at Sydney and the sample examined was not truly representative of the Bahrain water.

In addition, the pH of each food sample was determined and the food tested for its ability to support the growth of *V. cholerae*. The strain of *V. cholerae* used in these studies had been isolated from the faeces of a passenger on the London-Sydney flight. Each food sample was inoculated with approximately 500 *V. cholerae* per gram, incubated overnight at 28° C. and the number of *V. cholerae* counted on MacConkey agar. An incubation temperature of 28° C. was chosen because it approximated ambient temperature in Bahrain at the time of the outbreak. The results of these studies are given in Table 2.

Bacteriology of faeces

Faecal samples were collected from all passengers who disembarked in Australia and from all crew members associated with the flight at any stage of its journey from London to Sydney.

Faecal samples were generally examined within 1 hr. of collection, but where it was expected that delays of up to 6 hr. might occur, samples were also submitted

to the laboratory in alkaline peptone water. A long delay requiring the use of a transport medium (e.g. Cary-Blair, Stuart's or sea-salt medium) was experienced on only one occasion. In this case *V. cholerae* was isolated from a rectal swab submitted to the laboratory in Stuart's transport medium.

Because of the widespread dispersal of the passengers throughout Australia samples were examined at several laboratories. Although there is no standard procedure in Australia for the laboratory diagnosis of cholera, most laboratories followed the method laid down in 'Notes on the Laboratory Diagnosis of Cholera' (Commonwealth Department of Health, 1971).

In this laboratory, faeces were immediately inoculated into alkaline peptone water and plated directly on solid media. The tubes of alkaline peptone water were incubated at 37° C. for 6 hr. and subcultured on solid media and into a further tube of alkaline peptone water for overnight incubation. This was plated on solid media if the direct cultures and the 6 hr. alkaline peptone water subcultures were negative.

Initially five solid plating media were used; MacConkey agar, alkaline peptone agar, cholera medium (lauryl sulphate tellurite agar), Monsur's medium (alkaline taurocholate tellurite gelatin agar) and TCBS agar (thiosulphate citrate bile-salt sucrose agar). However, as the workload increased, it was necessary to restrict the number of media used and a combination of TCBS (highly selective) and MacConkey agar (less selective) was chosen.

Colonies suspected of being vibrios were subcultured into peptone water which, after 24 hr. incubation, was examined for motility and used to carry out the usual range of biochemical tests (Carpenter, Hart, Hatfield & Weeks, 1968).

In many instances it was possible to give a presumptive diagnosis by doing the 'string' test (Smith, 1970), oxidase test and slide agglutination with *V. cholerae* antiserum on colonies taken directly from the MacConkey agar plate. Slide agglutination tests were carried out using polyvalent, Inaba and Ogawa antisera.

All organisms identified as *V. cholerae* were tested for chick-cell agglutination, sheep-cell haemolysis and sensitivity to 50 μg Polymyxin B. and all were found to be *V. cholerae* biotype El Tor.

In New South Wales all persons with positive cultures were treated with tetracycline for 5 days, then after a period of 24 hr. during which no antibiotics were given, daily faecal samples were collected and examined. Patients were released from quarantine when three successive negative samples were obtained. In all cases, the first three cultures, collected after antibiotic therapy had ceased, proved to be negative. In five instances a follow-up specimen was collected 2–3 weeks after release from quarantine. All were negative.

Thirteen positive faecal specimens were also examined after standing at room temperature for 2 weeks and again after 1 month. Three of these gave positive results after 2 weeks, but only one sample was positive after 1 month. This specimen, which was a rice-water stool, was still positive after 6 months at room temperature – which varied from 10° C. to 42° C. during the storage period.

DISCUSSION

The outbreak of cholera described here demonstrates the ease with which the organism can be spread to several countries following the contamination of food or water. Fortunately, Australia is a non-receptive area and secondary cases did not occur.

Immunization against cholera is frequently used as a quarantine measure, to prevent the introduction of the disease into non-cholera areas. The results of the present episode indicate that, although immunization may play some part in preventing symptoms of the disease, it does not prevent healthy carriers of the organism entering a country and therefore has little value as a quarantine measure. A good system of sanitation and a high level of personal hygiene, both of which are present in Australia, are far better barriers to infection than immunization. This fact has been recognized for some time, and in the United States of America cholera immunization is no longer considered necessary for persons entering the country, even from a known cholera area.

Cholera is usually considered to be a water-borne disease, but, in this outbreak, the available evidence indicates that a food item served as part of a meal was the most likely vehicle of infection. Exactly how the food was contaminated is a matter for speculation. A carrier of *V. cholerae* may have been present in the kitchen at the time of the outbreak, as two such carriers were detected fourteen days later. However, the possibility of indirect contamination from vegetables (e.g. lettuce) or contaminated mixing bowls or implements cannot be excluded.

Outbreaks of food-borne disease on international aircraft, due to organisms other than *V. cholerae*, have been described in the past (W.H.O. 1972; Cent. Dis. Control 1971). International airline companies are justifiably proud of the elaborate measures they take in relation to mechanical checks on their aircraft to ensure the safety of their passengers. It is, therefore, surprising that little attention has been paid to the urgent need for up-grading the facilities of airport caterers. Mossel & Hoogendoorn (1971) carried out world-wide checks on 25 airport catering units and found that 50 % of toilets had no hand basins, 55 % had no soap or towel and 55 % no fly screens. Refrigeration was inadequate in 30 % of the catering units and in 20 % of the units food-preparation areas were not separated from the washing-up section.

There is a need for the introduction of a code of practice for the supply of food and water to aircraft. This might deal with such matters as the screening of employees, the provision of hand-washing and toilet facilities, the cleaning of kitchens and equipment, the general design of premises including the amount of refrigerated space to be provided, and the devising of guidelines for food hygiene education. Consideration might also be given to recording the chlorine content of water loaded on aircraft, either at the time of loading, or during flight. If the latter, a simple colour-comparator method should be used, which could be easily applied by aircrew or cabin staff. Menus should be revised to eliminate items particularly susceptible to contamination and therefore 'high risk' foods. A system of surveillance of the bacteriological status of meals served on aircraft could be insti-

tuted. Consideration might also be given to the practice of freezing and holding for 48 hr. a sample of each meal prepared in flight kitchens – this is done in New Zealand. The World Health Organization might arrange, possibly through the International Air Transport Association, the necessary co-operation between experts on food hygiene and representatives of the major airlines to formulate such a code of practice. If the recommendations made were realistic, there should be no difficulty in securing adoption of the code by the airline companies, or even in persuading governments to give the code legal sanction if this were thought desirable.

The preparation of this report has required the co-operation of many people throughout Australia but the author is particularly indebted for the help he has received from Drs R. R. Bull, J. M. Crotty, C. J. Cummins, A. S. Cumming-Thom, W. P. H. Dakin, J. A. Forbes, J. R. L. Forsyth, D. J. Howell, A. H. Humphreys, B. P. McCloskey, D. B. Travers, R. C. Webb, R. H. C. Wells, and Abu Zaid.

Qantas Airways gave every possible help in the investigation of the outbreak. That so much is known of the epidemiology is due to the whole-hearted collaboration of their Medical Department.

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Myxomatosis: some observations on breeding the European rabbit flea *Spilopsyllus cuniculi* (Dale) in an animal house

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SUMMARY

Rabbit fleas for use in Myxomatosis investigations have been successfully bred on rabbits in an animal house. The timing of emergence appeared to be governed by a biological timing control interacting with different forms of disturbance. Yield was found to be related to litter size, the time the doe and her kittens were removed from the nest, the number of fleas put onto a doe before littering and the mean ambient temperature to which the doe was exposed in the week pre-partum. The survival rate of fleas in storage was affected by temperature, the degree of crowding, moisture content of the containers, whether fleas were fed or unfed and the source of fleas in terms of emergence times.

INTRODUCTION

The European rabbit flea, introduced into Australia in 1966 (Sobey & Menzies, 1969) has been studied in wild rabbit populations (Williams, 1971; Williams & Parer, 1971; Sobey & Conolly, 1971; Williams, Fullagar, Kogon & Davey, 1973) where it has readily established. The usefulness of the flea in terms of rabbit control is still under investigation. Should the flea be regarded as useful it could be required in large numbers for general dissemination, and information relating to the breeding of the flea would be of value. The key to breeding this flea is the dependence of its reproductive cycle on hormone(s) produced by the pregnant rabbit doe (Mead-Briggs & Rudge, 1960). This finding has been elaborated by Mead-Briggs (1964) and Rothschild & Ford (1964, 1966). The extraordinary dependence of this flea on the rabbit is exemplified by the requirement of the presence of kittens for copulation to take place, as was shown by Mead-Briggs & Vaughan (1969).

The present paper describes a method of breeding rabbit fleas in an animal house and some observations on the variables affecting flea production.

EXPERIMENTAL

Rabbits

Randomly bred white domestic rabbits, albino or vienna (vv) were used. A pelleted feed and water were supplied *ad lib*, supplemented twice a week by green feed in the form of freshly cut oats or millet.

Table 1. *The effect on yield of adding 0·5 in. of soil to the bottom of the breeding nest box*

Times	Treatment	Total yield	No. weaned	Yield/kitten
1	Soil	11,805	25	472
	No soil	16,530	26	636
2	Soil	33,120	105	315
	No soil	14,100	51	276
3	Soil	8,875	16	554
	No soil	9,025	17	531

Fleas

The fleas used were descendants of an importation from England in 1966 (Sobey & Menzies, 1969). To ensure genetic diversity and to avoid selection for our particular animal house conditions, the breeding fleas for seeding on to pregnant does were a mixture collected over the emergence period 17–45 days post-partum. To further ensure genetic diversity fleas were periodically collected from the field, screened to ensure they were virus free and added to the breeding fleas. Fleas put on to a doe before littering are referred to as 'seed' fleas.

Addition of soil to nest-boxes

When the flea breeding began it was standard practice to cover the nest-box tray with 0·5 in. of clean soil. Since it involved extra work the necessity for the addition of soil came into question and some comparative tests were made; these were conducted at three different seasonal times as it was thought that soil might become important at times when yields were low. The results given in Table 1 suggested that the addition of soil does not affect yield and its use was discontinued.

Assessment of flea numbers

Counting large numbers of fleas was found to be tedious and time consuming. Volumetric measurements were made and the relationship of these to count is illustrated in Fig. 1. These measurements were made with active, freshly combed fleas. Stored fleas, often more sluggish and with their stomachs depleted of blood, occupied less volume than active fleas. Active fleas allowed to stand for 15 min. and read undisturbed, showed a markedly reduced volumetric reading; however, if held in the hand for reading and thus disturbed slightly, the volume returned to that observed prestanding. In the present study, conversions from volume to numbers of fleas were made using Fig. 1.

A general method used for breeding fleas

The following method was planned to accommodate the dependence of the breeding cycle of the flea on that of the rabbit. Does were palpated to confirm pregnancy and introduced into a breeding cage at approximately 9 days pre-partum at which time they were seeded with fleas. The number of fleas put onto a doe varied in general between 100 and 500. The breeding cage was supplied with a sheet metal nest-box, 20 in. long by 12 in. wide by 12 in. high. The nest-box had

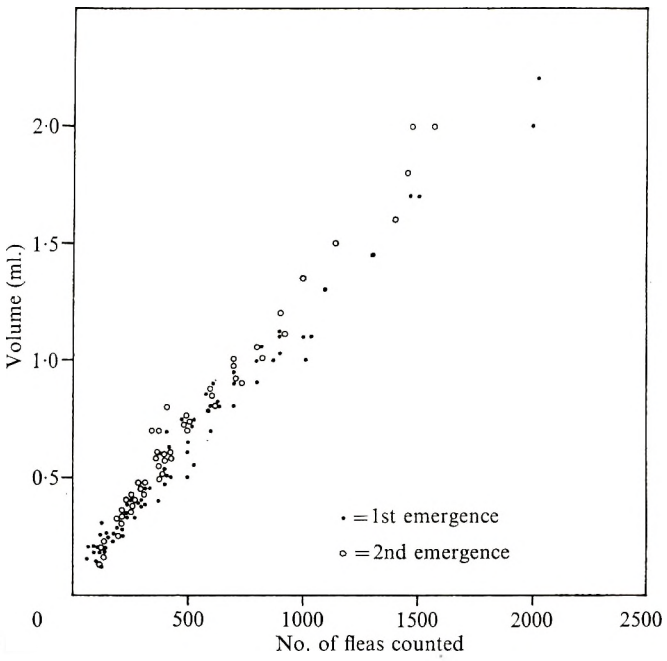

Fig. 1. The relationship of volume to number for freshly combed 1st and 2nd emergence fleas.

a detachable floor tray with a 3 in. high surround. The floor of the breeding cage and the nest-box tray were covered with straw to a depth of about 3 in. Litters were not counted until 5 days post-partum; the number then found was referred to as the number born. The doe and her kittens were removed from the breeding cage between 12 and 20 days post-partum, combed free of fleas and moved to a fresh breeding cage. The nest-box tray was then detached and either emptied into a large plastic bag or placed in a floor-pen containing a 'sweep' rabbit, usually an adult doe. Sweep rabbits were counted three times a week and the number of fleas was counted or measured volumetrically and recorded. The large plastic bags, 'bagged fleas', were emptied every 2 or 3 days into a large enamelled tray with 8 in. sides and the emerged fleas aspirated, counted and recorded.

The observations to be described cover two periods, the first when all nests were bagged and the second when fleas were collected via a sweep rabbit. The reason for changing from bags to sweep rabbits was to reduce the labour involved in flea collection. Variation in the rate of multiplication, in terms of fleas reaped divided by fleas seeded, over the total period of observation is illustrated in Fig. 2. During the period when nests were bagged as a routine, there was only limited temperature and humidity control in the animal house. Multiplication rate varied widely according to the time of the year, with the highest rates occurring between June and December and the worst, including many complete failures, during January to May. After 1971 with better temperature and humidity control, seasonal variation disappeared.

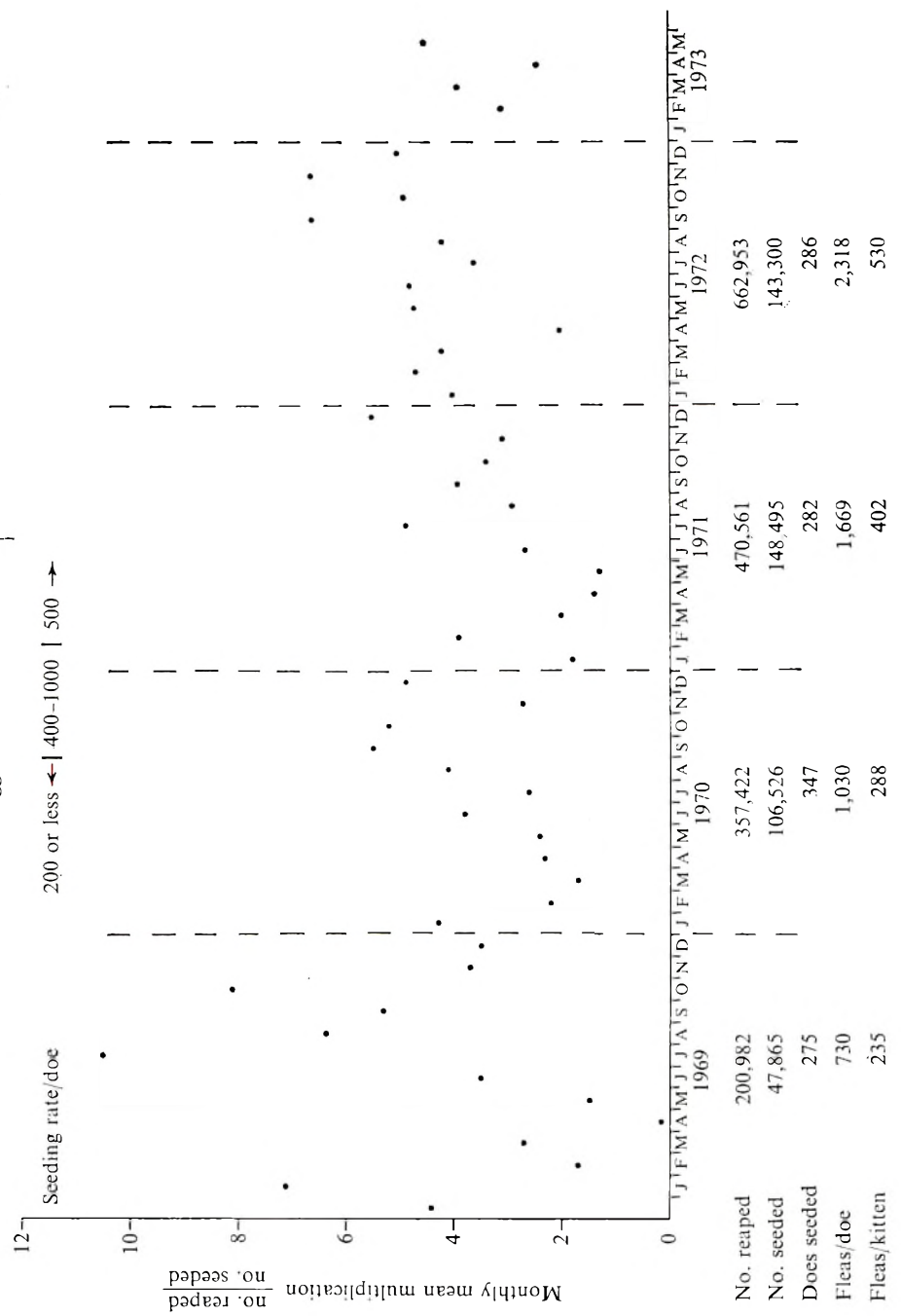

Fig. 2. Mean monthly multiplication rates, number of fleas seeded and reaped, and number of does seeded annually during 1969 to 1973.

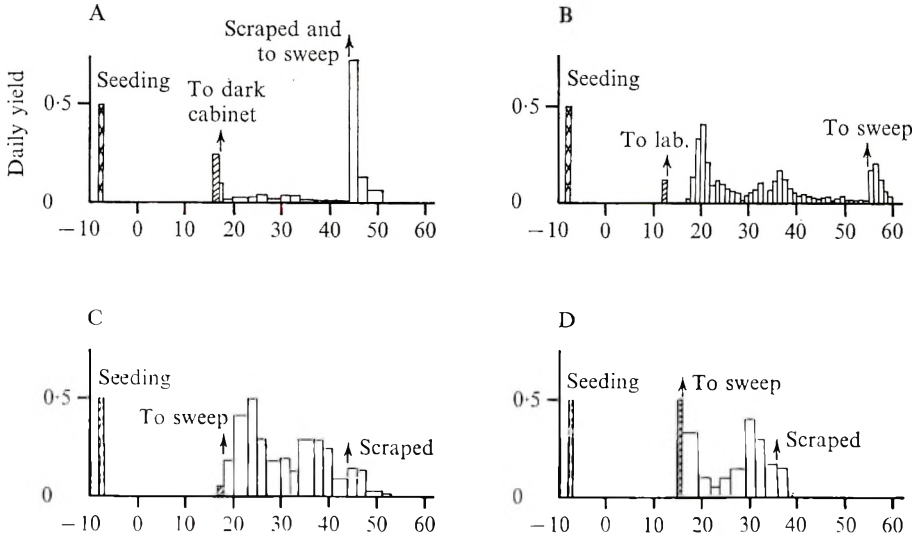

Fig. 3. Some examples of emergence patterns. (A) The nest in its tray was removed from the doe and her litter on the 17th day post-partum and placed in an undisturbed cabinet with a collecting bottle at the base. At 44 days the nest was scraped out of the nest-box tray into a floor pen housing a sweep rabbit. (B) The nest in its tray was removed to the laboratory at 23 days post-partum and suspended at room temperature in a polythene bag with a small opening into a collecting bottle. It was disturbed at least once daily by gentle movement of the straw from above and a few sharp blows underneath. At 55 days it was emptied into a floor pen with a sweep. Counts of emerging fleas were made daily. (C) The nest in its tray was placed in a floor pen with a sweep rabbit at 18 days post-partum and the sweep combed every 2–3 days. At 44 days the nest was scraped from its tray and returned to the sweep. The mean temperature for the week pre-partum was 19·5° C. (D) The nest in its tray was placed in a floor pen with a sweep rabbit at 16 days post-partum and the sweep combed every 2–3 days. At 36 days the nest was scraped from its tray and returned to the sweep. The mean temperature for the week pre-partum was 22° C.

Flea emergence

In general an early and a late emergence was found for both bagged fleas and fleas collected via a sweep. Some examples of the types of emergence patterns observed are illustrated in Fig. 3. In A the nest-box was removed from the doe and her litter on the 17th day post-partum. The tray of the nest-box was placed in a dark cabinet, with a collecting bottle in the base, and left undisturbed until 44 days post-partum when the nest was scraped out into a floor pen containing a sweep rabbit. Few fleas emerged until the nest was vigorously disturbed. In B the nest in its tray was removed to the laboratory at 12 days post-partum and suspended in a polythene bag with a small opening leading into a collecting bottle. The nest was disturbed at least once daily by gentle movement of the straw from above and a few sharp blows from below. At 55 days post-partum the nest was scraped into a floor pen containing a sweep rabbit. Counts of emerging fleas were made daily. With regular disturbances an emergence pattern with two main peaks was observed. The violent disturbances of scraping the nest out at 55 days post-partum resulted in further emergence of fleas. In C and D the nests in their trays were placed in two floor pens, each with a sweep rabbit. C was removed from the

Table 2. *Correlations between the time post-partum at which emergence peaks occurred and the mean ambient temperature to which does were exposed pre- and post-partum within the range of mean temperature* 18·9 *to* 23·3° *C.* (*min.* 15, *max.* 27). N *refers to the number of nests on which the correlation was based*

Emergence	1 week pre-	1 week post-	2 weeks post-	3 weeks post-
1st peak	−0·59	−0·48	−0·13	−0·20
N	40	43	36	34
2nd peak	−0·42	−0·18	−0·43	−0·28
N	38	41	33	31

doe and her kitten 18 days and D 16 days post-partum and the sweeps were combed every 2–3 days. Clearly the two main emergence peaks occur in the presence of continued rabbit disturbances. The difference between C and D in the timing of the emergence is possibly a result of the mean ambient temperature to which the does were exposed in the week pre-partum, C 19·4° C. and D 22·2° C. respectively. On the basis of the above data nests put to a floor pen and sweep rabbit were removed from the doe and her kittens at or about 17 days post-partum. Occasionally a nest was fouled by the doe with urine which raised the humidity, particularly in bagged nests. This resulted in a low yield at 2nd emergence and infestation of the fleas by different species of mites (*Acarus siro* Linnaeus, *Proctolaelaps* sp. Berlese, *Cheyletus malaccensis* Oudemans, and *Macrocheles* sp. Latreille). Fouling of the nest-tray by a sweep rabbit resulted in a low yield particularly among the late hatch fleas.

Temperature

Decreased mean ambient temperature increased the time post-partum that the emergence peaks occurred. Correlations between the time post-partum that the emergence peaks occurred and mean ambient temperatures to which does were exposed pre- and post-partum are shown in Table 2. The effect of temperature on the first emergence peak was greatest during the week pre- and the week post-partum, with the emergence peaks occurring later as the mean temperature decreased. Second peaks also occurred later at lower temperatures. An example of this peak shift with temperature is shown in Fig. 2C, D. The mean temperature to which the doe was exposed during the week before she littered was negatively correlated with yield ($r = -0{\cdot}31$ est. S.E. 0·1) within the range of mean temperature 18·9 to 23·3° C. (min 15°, max 27° C.).

Litter size

The correlation between yield and the number of kittens born was $r = 0{\cdot}33$ (est. S.E. 0·16) and between yield and the number of kittens weaned was $r = 0{\cdot}32$ (est. S.E. 0·16). The data are limited because when nests were first exposed to sweep rabbits for flea collection more than one nest was put to one sweep. Later when more floor pens became available single nests were given individual sweeps and the above data are based on single nests. Nests where all the kittens died generally failed to produce fleas.

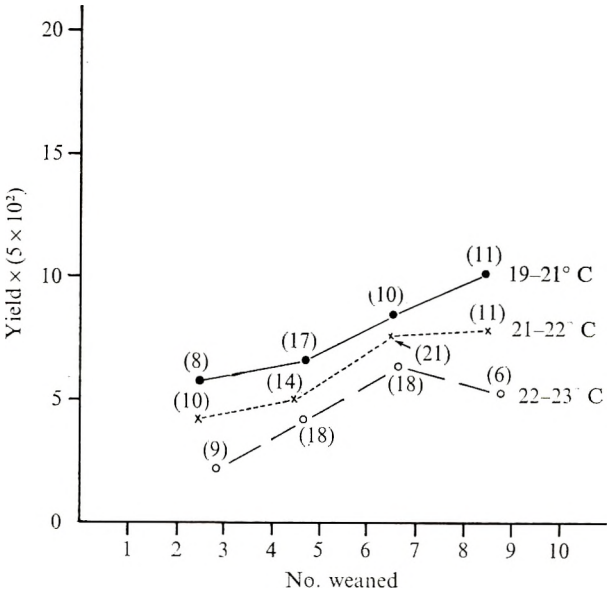

Fig. 4. Changes in mean yield for different mean numbers of kittens weaned for different mean ambient temperatures to which the does were exposed in the week pre-partum. ●, 19–21° C.; ×, 21–22° C.; ○, 22–23° C.

Table 3. *A comparison of the yield from nests in which no kittens were lost with those in which one was lost in the first week post-partum*

No. kittens born	No. lost in first week	No. of nests	$\bar{x}$ flea yield × (5 × 10²)
9, 10, 11	0	7	9·3
9, 10, 11	1	8	6·6
8	0	8	6·7
8	1	10	6·4
7	0	21	10·1
7	1	7	9·1
6	0	12	9·8
6	1	7	7·2
5	0	13	7·3
5	1	6	5·3
4	0	11	6·4
4	1	4	5·9

Only single nests were included during a time when yields were good.

The relationship of yield with the number of kittens weaned and the temperature to which the doe was exposed in the week before littering is illustrated in Fig. 4. Yield increased with increased number of kittens weaned and at each mean the yield was highest where the temperature to which the doe was exposed in the week pre-partum was lowest. There was a suggestion that at temperatures above 21° C. litters greater than six weaned have no advantage over those with six weaned.

The loss of a kitten in the first week post-partum was found to depress flea yield, as shown in Table 3. The loss of yield was consistent over the range of litter

Table 4. *The yield of fleas in relation to the number of fleas seeded onto does 9 days pre-partum*

	Seeding rate, fleas/doe					
	500		1,000		1,500	
	No. weaned	Yield	No. weaned	Yield	No. weaned	Yield
	5	798	4	1,410	8	2,380
	8	4,910	5	237	5	825
	6	4,150	4	3,698	8	2,305
	2	25	5	570		
	6	735	6	3,315		
	2	1,280				
	8	6,295				
	3	4,090				
	40	22,283	24	9,230	21	5,510
Yield/kitten		557		385		262
Yield/doe		2,785		1,846		1,836

sizes examined. Further, in comparisons of litters where no kittens were lost with litters which were reduced to the same size by the loss of a kitten, the litters without loss yielded more fleas on the average. In litters where all the kittens were lost between 5 and 10 days post-partum fleas were recovered from only a few nests. This observation must be qualified to the extent that many of these nests were discarded early, and might, had they been late yielding nests, have produced some fleas.

Seeding rate

Early experiments suggested that although lower seeding rates (100/doe) gave a higher rate of multiplication than higher rates (500/doe), the higher seeding rate produced more total fleas per doe and fewer complete failures. As fleas became more plentiful, seeding rates in excess of 500/doe were examined as a possible means of increasing flea production but they proved disappointing. The comparative yields from seeding rates of 500, 1000 and 1500 fleas per doe, are shown in Table 4. These data suggest that seeding rates in excess of 500 may confer some disadvantage to flea production as compared to 500 or less. This is further illustrated in Fig. 5 where the data suggest that the rate of multiplication decreases with increased seeding rate: further, yield per doe was highest and fewer nests failed to produce more than the number of fleas seeded, where 500 fleas were seeded. These data must be interpreted with some caution since the different seeding rates were not examined concurrently and all nests were not reaped in the same manner.

Storage of fleas

Allan (1956) was able to store fed and unfed fleas for nine months at $-6{\cdot}7^{\circ}$ C. Chapple & Lewis (1965) had 20% survive at 20 days and none at 42 days in a refrigerator at 4° C. In bottles stored underground they observed a 5% survival after 74 days. In the present work a temperature of 2° C. was selected as it was felt that temperatures between 2° and 4° C. would be readily available to workers

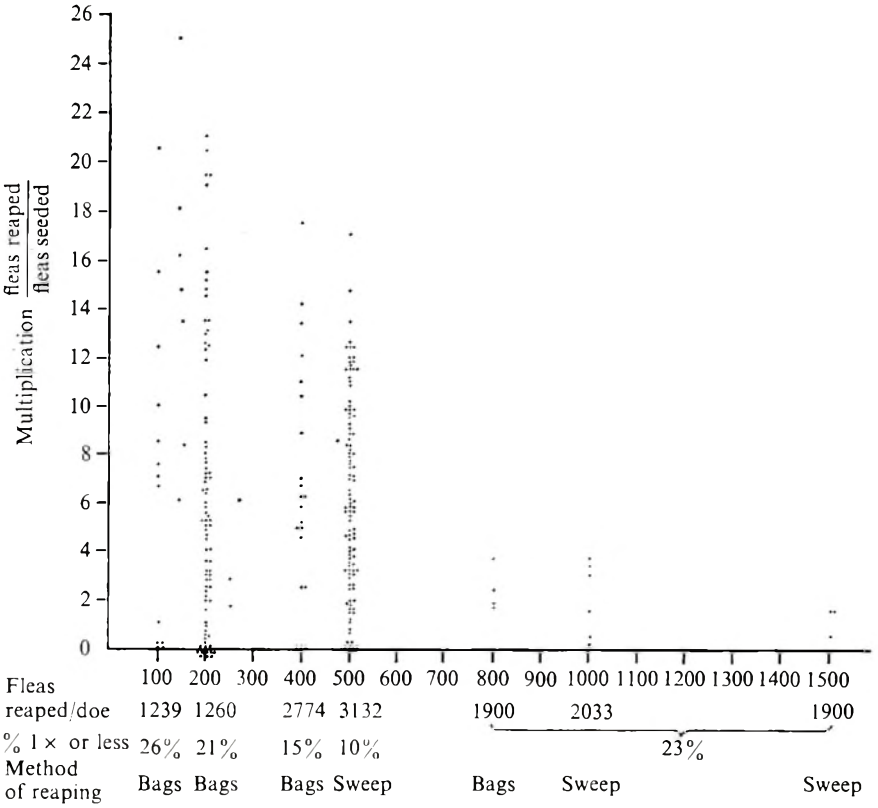

Fig. 5. Multiplication rates (fleas reaped/fleas seeded) observed for different numbers of fleas seeded.

producing fleas, whereas temperatures from 0 to $-10°$ C. might be difficult to obtain even if they proved better for flea storage.

It soon became apparent that the storage survival rate was very variable and affected by such things as size and type of container, degree of crowding, moisture content of containers, whether fleas were fed or unfed and the source of fleas in terms of emergence times.

In most of the work reported here, fleas were stored in 3 ml. flat-bottomed auto-analyser cups (plastic bottles) made of clear polystyrene and sealed with polyethylene caps. These tubes were initially selected because they were cheap and suitable as disposable containers for fleas being disseminated in the field. Their being plastic did not appear to confer any disadvantage. When compared with screw capped glass 1 oz. McCartney bottles with roughly equal numbers of fleas per volume, the 50 % survival time for the McCartney bottles was approximately 25 % lower than that for the plastic bottles. It was found that 100 fleas/3 ml. of container volume approached the 'overcrowding' limit for storage. With 150 or more/3 ml., fleas were, when removed from the cold storage, mostly dead, but on occasions anaesthetized, giving the appearance of being dead. We have observed apparently dead fleas show 100 % recovery after 2–3 hr. exposure to fresh air. Fleas from overcrowded bottles, whether or not they survived after storage, were observed to have their legs drawn up close to their bodies in contrast to fleas which

Table 5. *The percentage survival of fleas kept at room temperature and at 2° C. with and without the addition of moistened filter paper*

Treatment	% Survival 9 days at 22–24° C.		% Survival 56 days at 2° C.
	In light	In dark	
No filter paper	26	60	52
With filter paper added	25	33	30
With moist filter paper added	47	80	64

Fleas were stored in 3 ml. auto-analyser bottles at 20 fleas/bottle. Filter paper added was 1 cm.^{2} and where moistened approximately 10% by weight water added.

Table 6. *The half-life in days of fleas fed or unfed for different emergence times, stored at 2° C. in auto-analyser bottles at 100 fleas/bottle with no moisture or filter paper added*

Time of emergence	Half-life in days	
	Fed	Unfed
At or near 1st peak	55–65	60–70
Between 1st and 2nd peaks	50	—
At or near 2nd peak	70–80	90–120

Table 7. *The percentage survival of fleas from the peaks of 1st and 2nd emergence stored for 80 days at −1° C. or at 2–3° C.*

Origin of fleas	Treatment (° C.)	Sex	% Survival at 80 days	No. of fleas
1st Peak of Emergence	−1	♂♂	9	347
		♀♀	13	503
	2–3	♂♂	13	339
		♀♀	22	510
2nd Peak of Emergence	−1	♂♂	33	263
		♀♀	34	303
	2–3	♂♂	55	290
		♀♀	60	299

died from other causes where the legs were usually extended. The role of CO_2 or other toxic gases in this intoxication has not been determined.

To examine the effects of moisture on storage, 1 cm.^{2} pieces of filter paper with and without the addition of 10% water by weight, were added to the auto-analyser cups. The percentage survival for different treatments is shown in Table 5. Fleas were stored at the rate of 20 fleas/bottle. Dry filter paper reduced the percentage survival at both temperatures and the addition of water increased the survival. At room temperature (22–24° C.) fleas survived half as long again when kept in the dark than when kept in the light.

Unfed fleas, collected from bags, stored better than fed fleas combed from

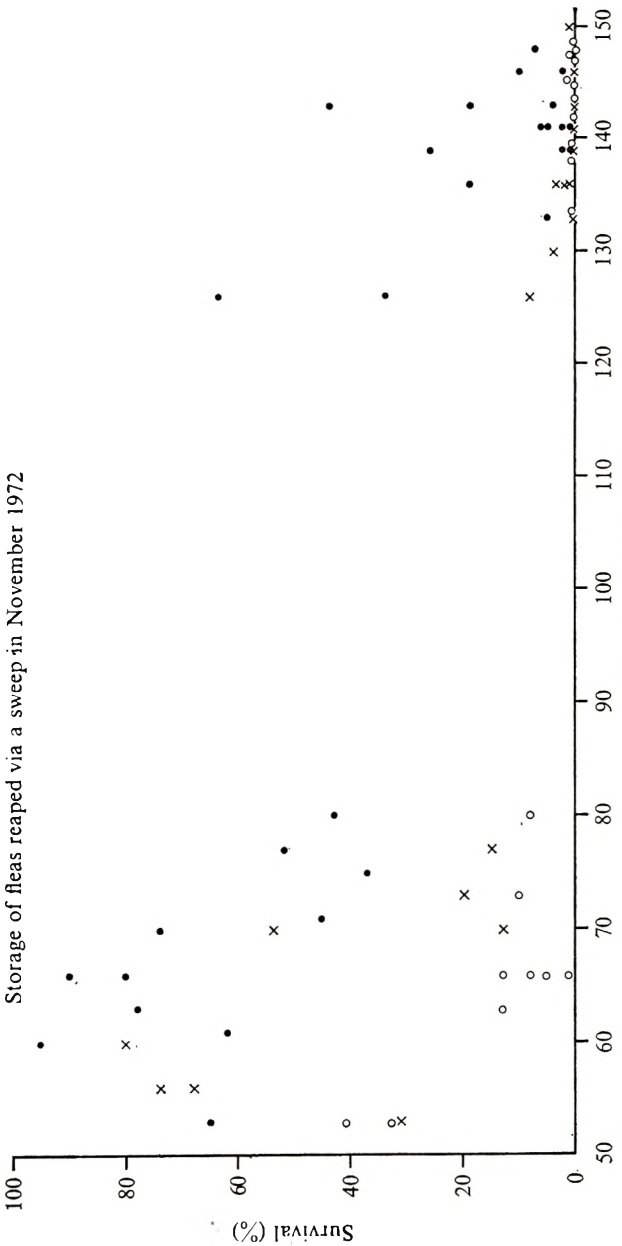

Fig. 6. The survival of fleas collected over the period of emergence and stored for different lengths of time. ×, At or near 1st peak; ●, at or near 2nd peak; ○, trough between peaks and early and late emergers.

sweeps, as shown in Table 6. Second emergence fleas stored better than 1st emergence fleas and fleas collected at times other than the main emergence times stored badly. The effect of emergence times is further illustrated in Table 7 where considerably more 2nd emergence fleas survived to 80 days than 1st emergence fleas. These data also suggest that storing fleas below 0° C. did not confer a storage advantage. Other data illustrating the storage advantage of 2nd emergence fleas are presented in Fig. 6. Fleas reaped in the emergence trough between 1st and 2nd emergence stored badly.

Occasionally fleas were left at 2° C. for long periods. One batch of 2nd emergence unfed fleas after 7 months had 11 % (20/177) survivors. With another batch of 2nd emergence unfed fleas, put on a rabbit after 10 months storage, 2 out of 1600 were recovered.

DISCUSSION

It is clear that fleas can be successfully bred in an animal house under quite wide ranges of temperature and humidity although control of these factors provides increased breeding efficiency. Our observations suggest that flea yield is greatly depressed by high humidity and maximum ambient temperatures in excess of 24° C. In the absence of adequate temperature and humidity control, seasonal variation in total yield was observed. It is interesting that the time of maximum flea yield, June to December, corresponded with the breeding season in the field suggesting a parallel response, possibly hormonal, of wild and domestic rabbits to the same seasonal effect to which the fleas respond. A clear seasonal effect on litter size and conception rate both negatively correlated with ambient temperature was reported by Sittmann, Rollins, Sittmann & Casady (1964). The effect of increased flea yield with decreased ambient temperature to which a doe is exposed in the week pre-partum, seems likely to be physiologically related to the apparent temperature effect on rabbit breeding efficiency.

The observations on emergence suggest that while fleas emerge in response to a disturbance of the nest there is a biological timing mechanism present which allows only part of the yield to respond early, 15–30 days post-partum, and the rest to respond after 30 days post-partum. This timing of emergence could be a survival mechanism related to the breeding behaviour of the rabbit. The fleas emerging before 30 days could leave the nest with the doe and the departing kittens whereas the later emerging fleas would be available for the following litter whether it followed immediately or after the break of the non-breeding season. The observation that late emerging fleas are better able to survive storage than early emerging fleas supports this idea. Second emerging fleas occupy a greater volume per flea than first emerging fleas (Fig. 1) either because they are bigger, or more active, or both, suggesting a physiological difference which could be related to their storage capabilities.

It seems likely that the correlation between the number of kittens in a litter and the yield of fleas may represent a causal relation in that the larger the litter, the greater the probability of high levels of sex hormones available to the fleas via both the doe and the kittens, ensuring a maximal ovarian maturation (Rothschild

& Ford, 1966). Further, the 'factor(s)' needed to bring the male rabbit flea to the correct 'physiological state' for mating (Mead-Briggs & Vaughan, 1969) and to ensure the adequate transfer of sperm (Rothschild, Ford & Hughes, 1970) is (are) probably quantitatively correlated with litter size.

Although the best total yield was achieved with a seeding rate of 500 fleas (Fig. 5) the maximum multiplication rate at this level of seeding, 17, was well below the maximum of 26 where the seeding rate was between 100 and 200 fleas. At seeding rates of 500 or more the maximum multiplication rate was only 5. The cause of this decreased multiplication rate with increased seeding is unknown.

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Preservation of corynebacteriophages by freeze-drying

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SUMMARY

A method of freeze-drying is described by which the activity of a series of corynebacteriophages was maintained over a test period of 30 months, whereas some of these strains became inactive if stored as liquid filtered lysates at 4° C., or frozen and held at −25° C.

INTRODUCTION

One of us (H.R.C.) has been engaged in an extended series of studies on bacteriophages carried by a number of pathogenic species of corynebacteria isolated from various lesions in man and animals. Observations on their morphological characters have recently been reported (Nagington & Carne, 1971).

The collection comprised some seventy phages, and for purposes of comparative study it was desired to be able to store multiple samples of individual phages for considerable periods without loss of activity. Preliminary observations indicated that some of these corynebacteriophages lost their activity relatively rapidly when held at 4° C. as liquid bacteria-free filtrates of lysed bacterial cultures. Considerable loss of titre also occurred when such filtrates were distributed in 0·25 ml. amounts in sealed glass ampoules and stored at −25° C. However, no loss of activity occurred if such ampoules were first rapidly frozen by immersion and then subsequently stored in liquid nitrogen at −196° C. This method proved excellent for limited numbers of samples, but because the large number of phages involved threatened to overtax the available liquid nitrogen refrigeration space, the possibility that freeze-drying might provide a satisfactory solution was then examined.

Davies & Kelly (1969) investigated the sensitivity of one of our phages to freezing and drying under different conditions and the method we have employed is based upon their findings.

MATERIALS AND METHODS

Bacteriophages

Fourteen phages were tested. These were derived from four different species of *Corynebacterium* isolated from infections in seven mammalian species as shown in Table 1.

Preparation of bacteriophage suspensions

Overnight cultures of sensitive indicator strains on tryptic digest agar slopes were washed off with 3 ml. of tryptic digest broth, shaken with glass beads in a high-speed mechanical shaker (H. Mickle, Hampton, Middlesex, England) for

Table 1. *Bacteriophages tested; lysogenic bacteria from which they were derived, and lesions from which the latter had been isolated*

Designation of phage	Lysogenic bacterium of origin	Mammalian species and type of lesion from which isolated
ov 1	*Corynebacterium ovis*	Sheep; caseous lymphadenitis
ov 2	*C. ovis*	Sheep; caseous lymphadenitis
ov 3	*C. ovis*	Sheep; caseous lymphadenitis
cap 1	*C. ovis*	Goat; caseous lymphadenitis
hq 1	*Corynebacterium* closely resembling *C. ovis*	Man; suppurative lymphadenitis
hq 2	*Corynebacterium* closely resembling *C. ovis*	Man; suppurative lymphadenitis
UH 3	*C. ulcerans*	Man; sore throat
uh 3	*C. ulcerans*	Man; sore throat
uh 5	*C. ulcerans*	Man; sore throat
uh 6	*C. ulcerans*	Monkey: sore throat
UB 1	*C. ulcerans*	Buffalo; ulcerative dermatitis
ub 2	*C. ulcerans*	Cow; mastitis
equi 4498	*C. equi*	Foal; suppurative pneumonia
pyog. 29	*C. pyogenes*	Cow; arthritis

2 min. to break up clumps. Sufficient of this suspension was then added to 100 ml. bottles of warmed (37° C.) tryptic digest broth to give a just perceptible opalescence. Bottles were then incubated for $1\frac{1}{2}$ hr. at 37° C. in a horizontal shaker giving 80 traverses per minute. Bacteria-free, high-titred phage suspensions were then added and the cultures shaken at 37° C. for a further 5 hr. After centrifugation at 10,000 rev./min. for 20 min. the supernatant was passed through Millipore filters (22 μm.) in all but two instances in which small volumes were passed through Membrane Filter No. 17 Oxoid.

The supernatant was dispersed in 0·25 ml. volumes into sterile 0·5 ml. freeze-drying ampoules (Johnson and Jorgensen, London), sealed, and rapidly frozen by immersion in liquid nitrogen, and then held in a liquid nitrogen refrigerator to await results of tests for freedom from bacteria and determination of the phage titre.

Filtrates were tested for freedom from bacteria by sowing liberally on appropriate culture media, and phage titres were determined by the Adams overlayer method (Adams, 1959).

Preparation of experimental samples

Appropriate numbers of ampoules of frozen, high-titre, bacteria-free lysate were rapidly thawed by agitation in water at 37° C. To the liquid phage suspension was then added an equal volume of a mixture of equal parts of 20% peptone, 10% sucrose and 2% sodium glutamate (sterilized by filtration). The phage-additive mixture was dispensed in 0·1 ml. amounts into sterile 0·5 ml. freeze-drying tubes (Johnson and Jorgensen, London) plugged with cotton wool, and the tubes were rapidly frozen by standing in a metal rack in a vacuum bowl containing liquid nitrogen. The frozen tubes were then transferred to the liquid nitrogen refrigerator until a convenient time to freeze-dry.

Table 2. *P.F.U./ml. of phages before freeze-drying, shortly after, and* 30 *months later after storage at* $-25°$ *C.*

Phage	At time of freeze-drying	3–15 days after freeze-drying	30 months after freeze-drying
ov 2	10^8	10^7	4×10^7
ov 3	10^{10}	(Not done)	10^{10}
cap 1	10^9	10^8	10^9
hq 1	3×10^9	10^{10}	10^8
hq 2	3×10^9	10^{10}	10^9
UH 3	10^8	10^7	10^8
uh 3	3×10^{10}	10^9	10^9
uh 5	2×10^{10}	10^{10}	10^9
uh 6	10^7	10^7	10^7
UB 1	10^{10}	4×10^9	10^{10}
ub 2	5×10^9	10^9	10^{10}
equi 4498	10^{10}	10^9	10^9

Freeze-drying

The apparatus used was that described by Greaves & Davies (1965). This comprised a two-stage thermoelectric refrigerator mounted on a thick brass plate which was water-cooled on the opposite side. A recessed 'O' ring in this plate gave a vacuum seal for the 'bell jar' top of the desiccator. The desiccator chamber was connected to a Cenco 'Megavac' pump via a vacuum valve and phosphorus pentoxide trap. An aluminium block, drilled to take 25 freeze-drying tubes, was placed empty in the centre of the dryer plate; the metal heat screen was placed in position and the bell jar placed over; refrigerator and pump were started and the temperature adjusted to $-35°$ C.

The apparatus was allowed to run for 30 min. to cool the aluminium block. The frozen tubes of phage suspension were then transferred from liquid nitrogen into the cavities of the block, and the apparatus started up again. After 30 hr. the temperature was raised to $-25°$ C. to increase the rate of drying and held at this for another 24 hr. The temperature was then allowed to come up to room temperature and held at this overnight. Tubes were then constricted, put on a manifold for secondary drying over P_2O_5 and after 4–6 hr. were sealed *in vacuo* and stored at $-25°$ C.

Dried samples were tested for phage titre by overlayer assays at 3 days and 30 months after freeze-drying. For this purpose the dried deposit in tubes was dissolved in 0·45 ml. of sterile 0·85% NaCl solution to give a 1/10 dilution, and from this further dilutions of 10^{-2} to 10^{-10} were prepared in tryptic digest broth for overlayer assays.

RESULTS

Table 2 gives the p.f.u./ml. of each phage, (*a*) just before freeze-drying, (*b*) 3 days after, and (*c*) 30 months after.

It will be seen that no significant loss of titre occurred as a result of the freeze-drying procedure used, and it remained essentially unchanged throughout a period of 30 months. The minor variations in titre are within the limits of experimental

variation with such phages which have a marked propensity to clump, probably because of the high lipid content of their bacterial hosts.

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The characterization of plasmids in the enterobacteria

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SUMMARY

The routine methods used in the Enteric Reference Laboratory for the study of enterobacterial plasmids are described. The results of their application to plasmids of diverse origin, and their value for the categorization of those plasmids, are presented and discussed.

INTRODUCTION

Plasmids in the enterobacteria mediate the transfer of a variety of genetic determinants, including those for drug resistance, haemolysin and enterotoxin synthesis, colicinogeny, heavy metal tolerance, resistance to ultra-violet irradiation, carbohydrate fermentation, H_2S synthesis and other metabolic characters. The drug resistances transferred include those to ampicillin, carbenicillin, cephalosporins, chloramphenicol, neomycin-kanamycin, streptomycin, sulphonamides, tetracyclines, gentamicin and trimethoprim.

Transfer systems have been characterized and classified by various methods in different laboratories. However, standard methods of plasmid characterization are needed to compare transfer systems from different sources. Such comparisons may ultimately furnish information about the phylogenetic relationships and origins of the plasmids concerned, and about their host bacteria.

In the Enteric Reference Laboratory (ERL), we have studied many transfer systems of enterobacteria from human and animal sources. These have been routinely characterized by the examination of the following properties:

1. The range of resistances in the wild host strain.
2. The range of resistances transferred from that strain to 'standard' recipient strains.
3. The possible multiplicity of plasmids present in a strain.
4. The presence and identity of colicinogeny.
5. The class of resistance factor (R factor) involved, that is, whether it belongs to Class 1, in which the resistance determinant and transfer factor are covalently bonded to form a single plasmid; or whether it is a Class 2 system, in which the resistance determinant (R determinant) and transfer factor form distinct plasmids, possibly associated only during transfer (Anderson, 1968, 1969; Anderson & Threlfall, 1970; Anderson & Natkin, 1972; Humphreys, Grindley & Anderson, 1972).

6. The transfer kinetics of the plasmid(s).

7. The mobilization of non auto-transferring (= non-transferring) resistance determinants in the strains by 'standard' transfer factors (Anderson, 1965*a*, *b*).

8. The determination of whether the plasmids enable their hosts to support multiplication of known donor-specific phages (Grindley & Anderson, 1971).

9. The compatibility group(s) of the plasmid(s) present in the wild strain (Hedges & Datta, 1971; Datta & Hedges, 1971; Chabbert *et al.* 1972; Grindley, Grindley & Anderson, 1972; Grindley, Humphreys & Anderson, 1973).

10. The fertility inhibition (*fi*) character (Egawa & Hirota, 1962; Watanabe & Fukasawa, 1962; Watanabe, Fukasawa & Takano, 1962; Watanabe, 1963; Watanabe *et al.* 1964).

11. Phage restriction in *Escherichia coli* K12 (= K12) and salmonellas (Anderson, 1966; Anderson, Threlfall, Carr & Savoy, 1973).

12. The degrees of drug resistance conferred by R factors or R determinants.

13. The molecular characteristics of the plasmid DNA.

In this article we shall describe the application of these methods of characterization to nine transfer systems.

MATERIALS AND METHODS

Media

Liquid media for the growth of bacterial cultures contained 20 g. Bacto dehydrated nutrient broth (Difco Laboratories) and 8·5 g. NaCl/l.; for solid media, 13 g./l. of Davis New Zealand powdered agar was added. Lactose and non-lactose fermenting cultures were differentiated by plating on Oxoid MacConkey agar.

Bacterial strains

The laboratory strains used in these studies are listed in Table 1.

Bacteriophages and phage-typing

The donor-specific phages for F- and I-specificity determination were $\mu 2$ (Dettori, Maccacaro & Piccinin, 1961) and If1 (Meynell & Lawn, 1968) respectively. The receptors for these phages are the F and I sex fimbriae respectively.

Phage restriction in K12 was tested with the 'female-specific' phage $\phi 2$ of Cuzin (1965) (Pitton & Anderson, 1970).

Phage restriction in salmonellas was investigated by phage-typing the respective strains. *S. typhimurium* was phage-typed by the methods of Callow (1959) and Anderson (1964, and in preparation); *S. typhi* carrying transfer systems was phage-typed according to Craigie & Yen (1938*a*, *b*) and Craigie & Felix (1947); and *S. paratyphi B* by the method of Felix & Callow (1943, 1951) and Anderson, (1964). The relevant techniques were summarized by Anderson & Williams (1956).

Drug resistance

Resistance to ampicillin (A), chloramphenicol (C), gentamicin (G), neomycin-kanamycin (K), streptomycin (S) and tetracyclines (T) was routinely tested for by a diffusion method on nutrient agar plates, using strips of blotting paper (Ford's

Table 1. *Laboratory strains used as donors and recipients*

ERL No.	Genotype	Plasmid carried	Designation
14R525	*E. coli* K12F$^-$*lac*$^+$Nx^r	—	K12
19R689	*S. typhimurium* phage type 36 Nx^r	—	Type 36
26R862	*S. typhi* Vi-phage type A Nx^r	—	*S. typhi* type A
B1363	*S. paratyphi B* phage type 1 var. 2	—	*S. paratyphi* B type 1 var. 2
RT641	*S. typhimurium* phage type 6 Δ$^+$	Δ	Type 6 Δ$^+$
1R380	*E. coli* K12F$^-$*lac*$^+$Δ$^+$	Δ	K12 Δ$^+$
22R149	*S. typhimurium* phage type 125 Str^{r}ColI$^+$	ColI	Type 125 ColI$^+$
22R81	*E. coli* K12F$^-$*lac*$^+$Str^{r}ColI$^+$	ColI	K12 ColI$^+$
18R951	*E. coli* K12F$^-$*lac*$^-$Str^{r}X$^+$	X	K12 X$^+$
27R207	*E. coli* K12HfrH Nx^r	F	K12HfrH
40R880	*E coli* K12 F$^+$	F	K12 F$^+$
20R770	*E. coli* K12 F$^-$*lac*$^+$T-Δ*drp*1$^+$	T-Δ*drp*1$^+$	K12 T-Δ*drp*1
38R778	*E. coli* K12 F$^-$*lac*$^+$ R-144-3$^+$	R144-3	K12 R144-3$^+$

Symbols

Nx^r = chromosomal resistance to nalidixic acid.
Str^r = chromosomal resistance to streptomycin.

Plasmid designation

Δ = Δ transfer factor (Anderson & Lewis, 1965*b*).
T-Δ = tetracycline R factor (Anderson & Lewis, 1965*b*).
ColI = ColI-P9 factor (Fredericq, 1956).
X = *fi*$^+$ F-like transfer factor from *S. typhimurium* 5M4136 (Anderson *et al.* 1968).
F = F factor.
T-Δ*drp*1 = derepressed mutant of T-Δ (Grindley & Anderson, 1971).
R144-3 = derepressed mutant of the I-like R factor R144 (Meynell & Datta, 1967). R144 was isolated from *S. typhimurium* 4M91, characterized in the Enteric Reference Laboratory in 1964 (Anderson & Datta, unpublished).

428 Mill) 80 mm × 7 mm impregnated with the respective drugs and freeze-dried. These strips were prepared in the Enteric Reference Laboratory. The cultures were streaked at right angles to the antibiotic strips, and control sensitive and resistant cultures were included in each test. This method not only detects resistance, but enables its magnitude to be roughly compared with that of other cultures on the same plate (see Plate 1). Resistance to sulphonamides and trimethoprim was detected by spotting 0·01 ml. drops of a 10^{-4} dilution of a late exponential phase broth culture of the test strain on nutrient agar containing 5 % v/v of lysed horse blood, and either 100 μg./ml. of sulphathiazole or 0·5 μg./ml. of trimethoprim (see Plate 2, Figs. 1 and 2). Sensitive and resistant controls were always included. Nalidixic acid resistance was also detected by this method, the nutrient agar containing 40 μg./ml. of the drug (see Plate 2, Figs. 3 and 4). Screening for furazolidone resistance was effected by spotting 0·01 ml. of undiluted culture of the strain on nutrient agar containing 100 μg./ml. of the drug.

Colicinogeny

Colicinogeny was detected by the agar-overlay method of Fredericq (1948), using K12 as the indicator strain. Colicins were identified on the basis of the

immunity of standard colicinogenic cultures to the lethal effects of their respective colicins, and of the resistance of known mutants of K12 to the action of specific colicins (Fredericq, 1948).

Transfer of plasmids

The conjugation techniques used were those of Anderson & Lewis (1965*a*, *b*). Resistance transfer was detected by plating mating mixtures, generally after overnight incubation, on agar plates containing the appropriate drugs. Drug-resistant donor strains of salmonellas in mating mixtures were suppressed with nalidixic acid (40 μg./ml.), when the recipient strains were nalidixic acid-resistant mutants, or by spreading salmonella phage O1 of Felix & Callow (1943) on the surface of the agar before plating the cross (Anderson & Lewis, 1965*a*, *b*). When the donor was K12 it was eliminated with colicin E2 (Anderson & Lewis, 1965*a*, *b*).

R determinant mobilization

The mobilizability of wild non-transferring R determinants was tested with the triparental cross for determinant mobilization (Anderson, 1965*a*, *b*). Three transfer factors were routinely used for this purpose in the primary donor strains: the *fi*$^-$ I-like Δ factor (Anderson & Lewis, 1965*b*); the *fi*$^-$ I-like ColI factor ColIb-P9; and the *fi*$^-$ F-like transfer factor X (Anderson, Pitton & Mayhew, 1968). As results with Δ and the ColI factor were identical, ColI is not shown in Table 4.

Phage multiplication

Plasmids were examined for their ability to enable host strains to propagate the donor-specific phages μ2 and If1 by the method of Grindley & Anderson (1971).

Determination of compatibility group

Plasmids were assigned to compatibility groups by examination of their ability to coexist with R factors of the known compatibility groups (Grindley *et al.* 1972).

Examination of fi *character*

This character was routinely identified by examining the inhibition of visible lysis by the F-specific phage μ2, which was spotted on surface cultures of K12F$^+$ and K12HfrH into which plasmids had been introduced (Pitton & Anderson, 1970). Factors that are *fi*$^+$ inhibit the synthesis of F fimbriae, which are the receptors for F-specific phages. Thus, when *fi*$^+$ factors are introduced into strains carrying the F factor, such as K12F$^+$ and K12Hfr, they reduce or abolish visible lysis by these phages. In contrast, *fi*$^-$ plasmids do not affect F-fimbrial synthesis, and therefore do not affect lysis of F$^+$ or Hfr strains by F-specific phages.

Isolation and measurement of plasmid DNA

Plasmid DNA was isolated, and the mean contour length (MCL) determined as described by Grindley *et al.* (1973). The molecular weight of the plasmids was calculated on the assumption that 1 μm. of DNA $= 2{\cdot}07 \times 10^6$ daltons (Lang, 1970).

Table 2. *Examination of the properties of transfer systems*

Property	Routine method of examination	Reference
Drug resistance	Strip diffusion tests for A, C, K, S, T and G	—
	Spot tests on solid media for Su, Tm, Fu and Nx	—
Colicinogeny:		
Production	Sensitivity of K12 to colicins	Fredericq (1948)
Identification	Immunity and resistance of standard strains	Fredericq (1948)
Transferability and frequency of transfer	Conjugation	Anderson & Lewis (1965*a*, *b*)
Mobilization of non auto-transferring resistance determinants	Triparental cross for determinant mobilization	Anderson (1965*a*, *b*)
F and I fimbrial synthesis	Donor-specific phage multiplication	Grindley & Anderson (1971)
Compatibility group	Ability to coexist with plasmids of defined groups	Grindley *et al.* (1972)
fi character	Inhibition of lysis of K12HfrH and K12F$^+$ by phage $\mu 2$	Pitton & Anderson (1970)
Phage restriction:		
In K12	Inhibition of lysis by phage $\phi 2$	Pitton & Anderson (1970)
In *S. typhimurium*	Phage-typing	Callow (1959); Anderson (1964)
In *S. typhi*	Phage-typing	Craigie & Yen (1938*a*, *b*); Craigie & Felix (1947)
In *S. paratyphi B*	Phage-typing	Felix & Callow (1943, 1951); Anderson (1964)
Plasmid DNA characteristics	Isolation and measurement of plasmid DNA	Grindley *et al.* (1973)
Degrees of drug resistance	Estimation of MICs of host strains to the respective drugs in liquid and on solid media	—

Drug resistance symbols: A, ampicillin; C, chloramphenicol; K, neomycin-kanamycin; S, streptomycin; Su, sulphonamides; G, gentamicin; T, tetracyclines; Tm, trimethoprim; Fu, furazolidone; Nx, nalidixic acid.

Minimal inhibitory concentration (MIC)

The MICs of strains carrying the ampicillin (A), chloramphenicol (C), streptomycin (S) and tetracycline (T) resistance determinants were estimated with doubling dilutions of the respective antibiotics in nutrient broth. A standard inoculum of approximately 10^2 bacterial cells/ml. was used. The MIC was the lowest concentration of antibiotic that inhibited visible growth of the test strain in nutrient broth. Kanamycin MICs were similarly determined in Mueller–Hinton broth. Attempts were made to measure resistance to sulphonamides (sulpha-

thiazole) on nutrient agar containing concentrations of the drug up to its limit of solubility (2000 μg./ml.). The resistance always exceeded this concentration.

The methods for the examination of the properties of transfer systems are summarized in Table 2.

The identification of the spectrum of activity of plasmid-determined enzymes such as penicillinases and those for inactivation of streptomycin, and the serological specificity of such enzymes, are also methods of plasmid characterization that can be added when necessary. As a general rule, we have routinely used only the differentiation of streptomycin adenylylation from phosphorylation by inclusion of spectinomycin in tests of streptomycin resistance. Spectinomycin is inactivated only by adenylylation.

RESULTS

The sources and designations of nine transfer systems used to exemplify the methods of characterization are shown in Table 3. Their properties are summarized in Table 4.

Drug resistance transfer

In the Δ transfer systems, first identified in *S. typhimurium* type 29 ASSuTFu, strain RT1 (Anderson & Lewis, 1965*a*, *b*), the Δ factor mediates independently the transfer of resistances to ampicillin, streptomycin-sulphonamides and tetracyclines. Furazolidone resistance has not yet been transferred.

The R factors TP110 (Anderson & Smith, 1972*a*) and TP102 (Grindley & Anderson, 1971), isolated from wild *S. typhimurium* strains 8M5251 and 8M5654, and the R factor TP114 (Grindley *et al.* 1972), from *E. coli* EC593, all carry a K determinant only; this codes for resistance to kanamycin, neomycin and paromomycin.

Factor 334 was isolated from a spontaneous kanamycin-sensitive segregant of *S. paratyphi B* type 3a var. 4, 7R334. This strain was first characterized in the ERL in 1964. R factor 334 transfers resistance to ampicillin, chloramphenicol, streptomycin and sulphonamides and, with the exception of loss of kanamycin resistance, is probably identical with the R factor R1, isolated from this strain by Meynell & Datta (1966).

R factor TP123, isolated from *S. typhi* 1T4739 (Anderson & Smith, 1972*b*), transfers resistance to chloramphenicol, streptomycin-sulphonamides and tetracyclines. 1T4739 is representative of the strain responsible for a widespread outbreak of chloramphenicol-resistant typhoid fever, which started in Mexico early in 1972 and was still active in 1973.

TP118 (Anderson & Threlfall, 1970) is an R factor identified in *S. enteritidis* strain E3538, belonging to phage type 8. It confers resistance to ampicillin and streptomycin.

Colicinogeny

The Ib colicinogeny determinant is covalently bonded to the transfer factor in TP110, and transfers with kanamycin resistance (= KColIb) (Anderson & Smith, 1972*a*). No identifiable Col determinants are associated with the remaining eight transfer systems.

Table 3. *Origin of transfer systems characterized*

Host strain: Serotype	Phage type	R-type*	ERL No.	Transfer system designation	Reference
S. typhimurium	29	ASSuTFu	RT1	A, SSu, T: resistance determinants	
				Δ transfer factor	Anderson & Lewis (1965*a*, *b*)
				A,Δ; SSu,Δ; T-Δ: Δ-mediated transfer systems	
S. typhimurium	104	K	8M5251	TP110	Anderson & Smith (1972*a*)
S. typhimurium	Untypable	K	8M5654	TP102	Grindley & Anderson (1971)
E. coli	.	K	EC593	TP114	Grindley *et al.* (1972)
S. paratyphi B	3a var. 4	ACSSu	7R334	334**	Pitton & Anderson (1970)
S. typhi	Degraded Vi-strain	CSSuT	1T4739	TP123	Anderson & Smith (1972*b*) Grindley *et al.* (1973)
S. enteritidis	8	AS	E3538	TP118	Anderson & Threlfall (1970)

Drug resistance symbols: see Table 2.

* R-type = spectrum of drug resistance.

** Isolated from a spontaneous kanamycin-sensitive segregant of strain 7R334, characterized in the Enteric Reference Laboratory in 1964. The transfer system is probably identical with that of R1, isolated from this strain by Meynell & Datta (1966).

Table 4. *Properties of transfer systems*

Properties	Resistance determinants		Transfer systems								
			Δ-mediated transfer systems								
	A	SSu	Δ (transfer factor)	A, SSu, Δ	T-Δ	TP110	TP102	TP114	334	TP123	TP118
Drug resistances transferred	.	.	.	A, SSu	T	K	K	K	ACSSu	CSSuT	AS
Colicinogeny	–	–	–	–	–	Ib	–	–	–	–	–
Class of R factor	.	.	.	2	1	1(KColIb)	1	1	1	1	1
Transfer frequency in overnight crosses*											
From original host to K12	0	0	5×10^{-1}	A 10^{-2} SSu 10^{-2} Δ 5×10^{-1}	10^{-6}	5×10^{-1}	10^{0}	5×10^{-1}	5×10^{-1}	10^{-4}	10^{-3}
From K12 to type 36	0	0	5×10^{-1}	A 10^{-3} SSu 10^{-3} Δ 5×10^{-1}	5×10^{-1}	5×10^{-1}	10^{0}	10^{-2}	10^{-4}	10^{-6}	10^{-4}**
From type 36 to K12	0	0	5×10^{-1}	A 10^{-2} SSu 10^{-2} Δ 5×10^{-1}	5×10^{-1}	5×10^{-1}	10^{0}	5×10^{-1}	5×10^{-1}	10^{-4}	10^{-3}***
Mobilization of non auto-transferring resistance by:											
fi⁻ I-like factor Δ	+	+	.	.	.	.	.	.	.	.	.
fi⁺ F-like factor X	–	–	.	.	.	.	.	.	.	.	.
Supports multiplication of 'male' phages:											
If I	.	.	+	+	+	+	+	+	–	–	–
$\mu 2$	.	.	–	–	–	–	–	–	+	–	–
Compatibility group	.	.	I_1	I_1	I_1	I_1	I_1	I_2	F_{II}	H	N
fi character	.	.	*fi*⁻	*fi*⁻	*fi*⁻	*fi*⁻	*fi*⁺	*fi*⁻	*fi*⁺	*fi*⁻	*fi*⁻
Phage restriction:											
in K12 (phage $\phi 2$)	–	–	+	+	+	+	–	–	–	–	–
in salmonellae (typing phages)	+	–	+	+	+	+	–	–	–	–	–

Table 4 (*cont.*)

Properties	Resistance determinants: A	Resistance determinants: SSu	Transfer system: Δ-mediated transfer systems: Δ (transfer factor)	Δ-mediated transfer systems: A, SSu, Δ	Δ-mediated transfer systems: T-Δ	TP110	TP102	TP114	334	TP123	TP118
Resultant phage type in:											
S. typhimurium type 36	125	NM	6	Δ⁺6	6	125	NM	NM	NM	NM	NM
S. typhi Vi-type A	ND	ND	Resistant to all Vi-phages	Δ⁺ resistant to all Vi-phages	Vi-type 29 Vi-phages I, IV, V, VI, VII restricted	Restricts Vi-phages III, V, VI, VII	NM	NM	NM	NM	NM
S. paratyphi B type 1 var. 2	ND	ND	Beccles var. 2	Δ⁺ Beccles var. 2	Beccles var. 2	NM	NM	NM	NM	NM	NM
MIC (μg/ml) in:											
Original host	A 3000	S 2000	.	A 3000 S 2000	T 250	K > 10,000	K > 10,000	K 1250	A 1000 C 500 S 125	C 150 S 32 T 125	A 500 S 125
K12	A 3000	S 250	.	A 3000 S 250	T 125	K > 10,000	K > 10,000	K 1250	A 1000 C 500 S 62·5	C 150 S 32 T 125	A 500 S 125
Type 36	A 3000	S 1000	.	A 3000 S 1000	T 250	K > 10,000	K > 10,000	K 2500	A 1000 C 500 S 125	C 150 S 32 T 125	A 500 S 125
DNA characteristics: MCL of plasmid (μm)	2·70	2·74	28·7	A 2·70 SSu 2·74 Δ 29·5	32·3	31·3	26·3	19·7	26·2	59·5	13·2
Molecular weight (daltons × 10^{-6})	5·6	5·7	59	A 5·6 SSu 5·7 Δ 61	67	64·8	54·4	40·8	54·2	123·2	27·3

Drug resistance symbols, see Table 2. Plasmid designation, see Table 3. Phage designations: If1, I-specific phage; μ2, F-specific phage.
* All frequencies are approximate. ** Transfer frequency to *S. enteritidis* type 1 (see text). *** Transfer frequency from *S. enteritidis* type 1 to K12.
MCL, mean contour length; NM, unchanged; ND, not determined.

Class of R factor

The tetracycline resistance determinant and Δ factor of the Δ-mediated transfer system are covalently bonded to form a single plasmid which is transmitted as an intact linkage group, the Class 1 R factor T-Δ. A,Δ and SSu,Δ are Class 2 resistance transfer systems in which the A and SSu resistance determinants and the Δ transfer factor are independent of each other in the host cell. The plasmids of a Class 2 system may be transmitted simultaneously or separately (Anderson & Lewis, 1965*a*, *b*; Anderson, 1968, 1969). The Δ factor is necessary for the transfer of the A and SSu determinants.

TP110, TP102, TP114, 334, TP123 and TP118 are Class 1 R factors. The R determinants and transfer factors are transmitted together in these transfer systems. The respective R determinants have not been found separately in exconjugants, although a low percentage of recipient cells may acquire only the transfer factor (Anderson & Lewis, 1965*b*; Anderson, 1966).

Transfer kinetics

Since overnight crosses are routinely used in these investigations, transfer frequencies are estimated as a fraction of the total recipient population at the termination of the cross. All frequencies quoted are near approximations. The plasmids Δ, T-Δ, TP110 and TP102, all transfer at high frequencies, up to 10^0, in overnight crosses. The A determinant of A,Δ transfers at lower frequency (10^{-2}) than that of Δ alone (up to 10^0) (Anderson & Lewis, 1965*b*). The SSu determinant of RT1 behaves similarly to A. TP114 transfers at 5×10^{-1} from *E. coli* EC593 to K12, and from K12 to type 36 at 10^{-2} or less. Type 36 transfers TP114 to K12 at 5×10^{-1}.

Factor 334 transfers at 5×10^{-1} in crosses from *S. paratyphi B* 7R334 and from type 36 to K12, but at only 10^{-4} from K12 to salmonella hosts.

TP123 transfers to K12 at 10^{-4} in crosses from *S. typhi* 1T4739 and type 36 respectively, and at 10^{-6} or less from K12 to *S. typhi* and type 36.

TP118 is relatively host specific: without modification this R factor will transfer only from *S. enteritidis*, in which it was first identified, to *S. enteritidis* (10^{-4}) or to K12 (10^{-3}) and from K12 to *S. enteritidis* (10^{-4}) (Anderson & Threlfall, 1970). Transfer of N group plasmids from K12 to *S. typhimurium* often occurs at very low frequencies. For example, TP120 (Grindley *et al.* 1972, 1973) which has the resistance spectrum ASSuT, transfers to *S. typhimurium* 36 at about 10^{-6} in overnight crosses. TP120, and other group N plasmids isolated during the same period in 1962 (Anderson & Datta, 1965) may lose resistance markers during transfer to *S. typhimurium*. Thus, TP120 may lose T or S during such transfer (Anderson & Janet White, unpublished observations).

Mobilization of non-transferring resistance determinants

The A and SSu determinants of the Δ transfer systems are plasmids that can be mobilized by I-like factors such as the Δ transfer factor and the ColI factor, but not by the F-like transfer factor X. Thus, there is some specificity in determinant-transfer factor associations (Anderson, 1968).

Host specificity may be an important character of some plasmids. For example, we have so far been unable to demonstrate transfer of I_1 plasmids to *Proteus mirabilis* PM1 (Anderson and Deniset, unpublished observations), although a number of F-like factors can enter that host.

Donor-specific phage multiplication

The transfer systems tested were grouped under three headings, as shown below:

1. F-like factors, which code for F sex fimbriae, thereby enabling their hosts to propagate the F-specific phages. R factor 334 belongs to this category.
2. I-like factors, which code for I sex fimbrial synthesis, enable their hosts to support multiplication of the I-specific phage If1. Examples are the transfer factor Δ and the R factors T-Δ, TP110, TP102 and TP114.
3. Transfer systems that do not confer on their hosts the ability to propagate either F-specific or I-specific phages; for example, TP123 and TP118. This category is heterogeneous.

Compatibility groups

Transfer systems have been divided into compatibility groups (Romero, 1970; Khatoon & Iyer, 1971; Hedges & Datta, 1971; Datta & Hedges, 1971; Chabbert *et al.* 1972; Grindley *et al.* 1972, 1973). In general, the members of each group are compatible with those of other groups, but are incompatible with each other.

As Table 4 shows, the I-like factors have been divided into the I_1 and I_2 compatibility groups. Δ, TP110 and TP102 are incompatible with other I-like plasmids such as R144-3, and belong to the group designated I_1 by Grindley *et al.* (1972). However, TP114, which codes for the synthesis of I fimbriae, is compatible with the I_1 factor T-Δ, and is the prototype of the I_2 group (Grindley *et al.* 1972).

The F-like R factor 334 is an F_{II} plasmid which is compatible with F_I factors such as the original F transfer factor (Hedges & Datta, 1972). TP123 belongs to the H group (Anderson & Smith, 1972*b*; Grindley *et al.* 1972, 1973), and TP118 to the N group (Grindley *et al.* 1972, 1973).

The fertility inhibition (fi) *character*

The I_1 plasmids Δ, T-Δ and TP110, and the I_2 plasmid TP114, are *fi*$^-$: they do not inhibit visible lysis of strains of K12F$^+$ or K12HfrH by the F-specific phage $\mu 2$. However, the I_1 R factor TP102 is *fi*$^+$; it reduces lysis by phage $\mu 2$ and transfer of chromosomal characters by K12HfrH (Grindley & Anderson, 1971).

The F_{II} R factor 334 is *fi*$^+$ (Pitton & Anderson, 1970). Most wild F-like factors show this character.

The H group plasmid TP123 and the N group plasmid TP118 are *fi*$^-$.

Phage restriction

*In K*12.

Of the I_1 factors examined, the *fi*$^-$ plasmids Δ, T-Δ and TP110 all reduce visible lysis of K12 by phage $\phi 2$. When the K12 strain carrying these factors is also F$^+$ or Hfr, visible lysis by phage $\phi 2$ is abolished. In contrast, the *fi*$^+$ I-like

R factor TP102 is non-restricting for this phage. TP114, the *fi*$^-$ plasmid of the I_2 group, is also non-restricting for ϕ2 in K12, as are the *fi*$^+$ F_{II} R factor 334 and the H and N plasmids TP123 and TP118 respectively.

In S. typhimurium *type* 36, S. typhi *Vi-type A and* S. paratyphi B 1 *var.* 2.

Δ *transfer systems.* A is one of the rare non auto-transferring resistance determinants so far observed to cause phage restriction (Anderson *et al.* 1968). It restricts phages 12 and 13 of the *S. typhimurium* typing scheme, thereby converting type 36 into type 125 (Anderson, 1966; Anderson *et al.* 1968). The SSu determinant does not cause phage restriction.

The Δ transfer factor and the T-Δ R factor restrict the lysis of type 36 by 24 of the 30 *S. typhimurium* typing phages, to produce type 6 (Anderson & Lewis, 1965*b*). They convert *S. paratyphi B* 1 var. 2, which is sensitive to all 11 of the *S. paratyphi B* typing phages, into phage type Beccles var. 2, sensitive to only 3 of the phages (Anderson, 1966). However, Δ differs from T-Δ in that it inhibits lysis of *S. typhi* type A by all 96 Vi-typing adaptations of Vi-phage II, whereas T-Δ converts type A into type 29, which is sensitive to 13 of the adaptations (Anderson, 1966). Moreover, Δ also restricts lysis of *S. typhi* by the unadapted Vi-phages I, III, IV, V, VI and VII, whereas *S. typhi* type A carrying T-Δ remains sensitive to Vi-phage III (Anderson, 1966).

*TP*110 *KColIb.* TP110 converts type 36 of *S. typhimurium* into type 125, and is a representative of the Γ group of transfer systems (Anderson *et al.* 1973). This R factor does not alter the Vi-type of *S. typhi* type A, but restricts the unadapted Vi-phages III, V, VI and VII of that host. It does not affect the phage type of *S. paratyphi B* 1 var. 2.

Other transfer systems. The I-like factors TP102 and TP114, the F_{II} R factor 334, the H plasmid TP123 and the N plasmid TP118, are all non-restricting in *S. typhimurium* type 36, *S. typhi* type A and *S. paratyphi B* 1 var. 2.

Degrees of drug resistance

Δ *systems.* The penicillin MIC of the wild host strain of *S. typhimurium* type 29, RT1, and of both type 36 and K12 carrying the A determinant, is 3000 μg./ml. (Anderson & Lewis, 1965*a*; Anderson *et al.* 1968). The streptomycin MIC is 2000 μg./ml. in RT1, 1000 μg./ml. in type 36, and 250 μg./ml. in K12. The tetracycline MIC of strains carrying T-Δ is 250 μg./ml. in the original host and in type 36, and 125 μg./ml. in K12. Plasmid-borne tetracycline resistance is commonly of this magnitude.

*TP*110, *TP*102 *and TP*114. *Kanamycin resistance.* The kanamycin resistance in strains of *S. typhimurium* and K12 carrying TP110 and TP102 is greater than 10,000 μg./ml. In contrast, the MIC of kanamycin in strains carrying TP114 is 1,250 μg./ml. in both the original *E. coli* host and K12, and about 2,500 μg./ml. in type 36.

R factor 334 *ACSSu.* The ampicillin MIC of the original strain of *S. paratyphi B* carrying 334 is 1000 μg./ml.; it is about the same in K12 and type 36. The chloramphenicol MIC of these strains is about 500 μg./ml. The streptomycin MIC is

125 μg./ml. in *S. paratyphi B* and *S. typhimurium*, and 62·5 μg./ml. in K12. All host strains are resistant to at least 2000 μg./ml. of sulphathiazole, the limit of solubility of the drug.

*TP*123 *CSSuT*. TP123 confers a chloramphenicol MIC of 150 μg./ml. on its original host strain of *S. typhi* (Anderson & Smith, 1972*b*), and on K12 and type 36. Its host strains also have MICs of 32 μg./ml. to streptomycin, and 125 μg./ml. to tetracycline. The sulphonamide MIC exceeds 2000 μg./ml.

*TP*118 *AS*. The ampicillin MIC of *S. enteritidis*, *S. typhimurium* and K12 carrying TP118 is 500 μg./ml., and the streptomycin MIC, 125 μg./ml.

DNA characteristics

The DNA molecules of factors of a given compatibility group are of similar size, and usually of similar composition (Grindley *et al.* 1973). The group I_1 factors, Δ, T-Δ, TP110 and TP102 have mean contour lengths (MCLs) of 28·7, 32·3,* 31·3 and 26·3 μm. respectively, corresponding to molecular weights of 59, 67, 65 and 54×10^6 daltons. The prototype of the I_2 group is smaller, with a MCL of 19·7 μm. and a molecular weight of 41×10^6 daltons. Although it codes for I fimbriae, it is distinct from plasmids of the I_1 group.

The F_{II} R factor 334 has a contour length of 26·3 μm., corresponding to a molecular weight of 54×10^6 daltons. The contour length of the H group factor TP123 is 59·5 μm. and its molecular weight 123×10^6 daltons. The N group R factor TP118 is 13·2 μm. long, with a molecular weight of 27×10^6 daltons.

The non auto-transferring resistance determinants A and SSu are plasmids which are distinct from each other and from the compatibility groups described above. The mean contour length of A is 2·70 μm., and that of SSu 2·74 μm., corresponding to molecular weights of $5{\cdot}6 \times 10^6$ and $5{\cdot}7 \times 10^6$ daltons respectively. Such determinants are present in multiple copies per chromosone, whereas only one copy of transferable plasmids is found.

DISCUSSION

We have described the properties of nine transfer systems in salmonellas and *E. coli* carrying resistance to various drugs. These systems have been divided into two classes according to the relationships between the R determinants and the transfer factors (Anderson, 1968, 1969; Anderson & Threlfall, 1970; Anderson & Natkin, 1972; Humphreys *et al.* 1972). Seven of the transfer systems, T-Δ, TP110, TP102, TP114, 334, TP123 and TP118, are Class 1 R factors, that is, they are single covalently bonded plasmids which occupy the cellular attachment site of the respective transfer factor, and are transferred as intact linkage groups. Two, the A,Δ and SSu,Δ systems, belong to Class 2, in which the R determinants and the Δ transfer factor are distinct molecules, independent of each other and occupying separate attachment sites in the host cell: they are possibly associated only during transfer to a new host.

The R factor TP110 is a Class 1 system in which the kanamycin resistance

* The difference between the contour lengths of Δ and T-Δ, 3·6 μm., gives an approximate length for the T resistance determinant.

determinant, the ColIb determinant and the I-like transfer factor are covalently bonded to form a single plasmid. ColI determinants are usually found in wild strains recombined with I-like transfer factors, and are common in *S. typhimurium*. Other Col determinants, such as E1 and E2, occur as independent non auto-transferring plasmids, analogous to A and SSu (Anderson & Lewis, 1965*b*).

Transferable plasmids can also be categorized according to the nature of the sex fimbrial synthesis they encode and by compatibility studies. Thus Δ, T-Δ, TP110 and TP102 are plasmids of the I_1 compatibility group, while TP114, although it supports multiplication of phage If1, is compatible with I_1 plasmids and is the prototype of the I_2 group (Grindley *et al.* 1972). R factor 334 is an F_{II} plasmid, TP123 belongs to group H (Anderson & Smith, 1972*b*; Grindley *et al.* 1972) and TP118 to group N.

Molecular characterization of plasmid DNA confirms the groupings obtained by genetic analysis. The contour lengths of plasmids of the I_1 group are similar (average contour length 29·7 μm.) and differ substantially from that of the I_2 plasmid TP114 (contour length 19·7 μm.). The contour lengths of TP118, the N group plasmid (13·2 μm.), and TP123, the H plasmid (59·5 μm.), are also quite distinct from each other and from I_1 and I_2 plasmids (Grindley *et al.* 1973).

DNA reassociation of high degree usually occurs between plasmids of a single compatibility group, but not between those of different groups (Grindley *et al.* 1973). For example, reassociation readily takes place between the DNA of different I_1 plasmids, but not between plasmids of this group and the DNA of the I_2 plasmid TP114, or that of plasmids of other compatibility groups. However, some plasmids may be atypical. Thus, no reassociation could be demonstrated between the DNA of TP116, an H group R factor, and that of other H group plasmids (Grindley *et al.* 1973). Incompatibility, therefore, may not necessarily indicate molecular similarity.

Plasmids of a given compatibility group have similar transfer kinetics, which often differ from those of other groups. For example, the I_1 plasmids we have studied invariably transfer at high frequencies (up to 10°) in overnight crosses, whereas the H plasmid TP123 transfers at low frequencies, about 10^{-4} into K12, and as low as 10^{-6} into *S. typhimurium* and *S. typhi*. These low transfer frequencies are a feature of all H group plasmids so far examined.

It is worth noting that members of the same compatibility group may carry different resistance determinants in a Class 1 association, and, conversely, that members of different groups may carry determinants coding for similar resistances. Resistance markers are therefore of limited value in the identification of R factors. Thus, the Class 1 R factor in the strain of *Shigella dysenteriae* 1, which caused the huge dysentery outbreak in Central America from 1968 onwards, codes for resistance to chloramphenicol, streptomycin, sulphonamides and tetracyclines, and belongs to compatibility group B. The strain of *S. typhi* that caused the widespread typhoid outbreak in Mexico in 1972 is resistant to the same drugs, but belongs to group H (Grindley *et al.* 1972). These two R factors are thus quite distinct from each other, despite the similarity of their resistance markers.

The *fi*$^+$ character (Egawa & Hirota, 1962; Watanabe & Fukasawa, 1962; Watanabe, 1963; Watanabe *et al.* 1962, 1964) has now been identified in several

groups of plasmids. Although most I_1 and N factors are *fi*$^-$, *fi*$^+$ factors of these groups have been identified (Grindley & Anderson, 1971; Grindley *et al.* 1973). However, all wild F_{II} R factors so far examined are *fi*$^+$.

Plasmids within a single compatibility group can be subdivided by their phage-restrictive effects in K12 and in salmonellas. *fi*$^-$ I-like R factors and transfer factors have been divided into eleven types by their typing phage restriction in *S. typhimurium* (Anderson *et al.* 1973). Further subdivision may be possible by the determination of phage restriction in *S. typhi* and *S. paratyphi B* (Anderson, 1966). The F factor of K12 restricts one *S. typhimurium* typing phage (Anderson *et al.* 1973) and unadapted Vi-phages III, V, VI and VII of the *S. typhi* Vi-typing scheme.

Non-transferring resistance determinants can be characterized by the specificity of their mobilization by transfer factors: for example, SSu determinants are in general most easily mobilized by I-like factors. The degrees of resistance they confer on their host strains may also be characteristic. Only resistance determinants from Class 2 systems can be characterized by their mobilization specificity, since they can be isolated without transfer factors in the host cell.

The foregoing description outlines the methods currently used in the Enteric Reference Laboratory for the characterization of transfer systems in the enterobacteria. These methods are useful for classifying the systems on the basis of their genetic properties and molecular structure, and may ultimately expose their origins and host relationships in man and animals.

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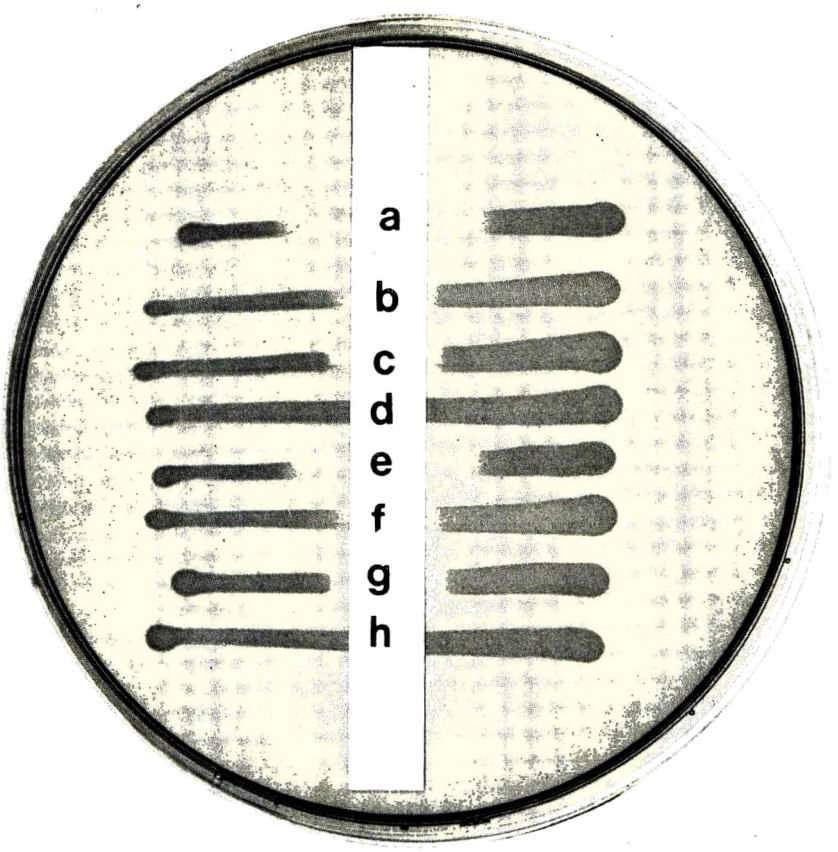

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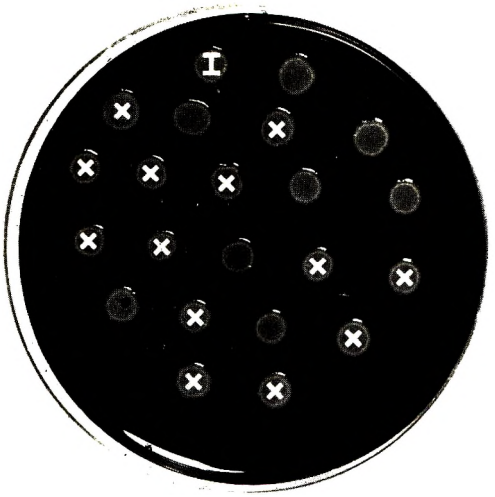

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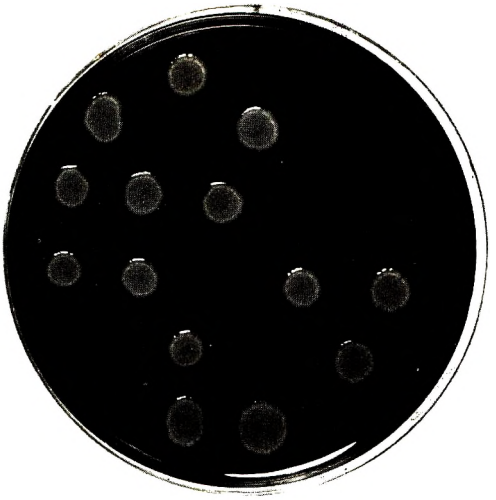

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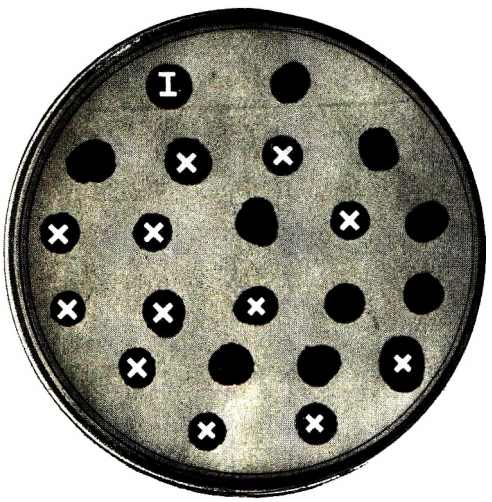

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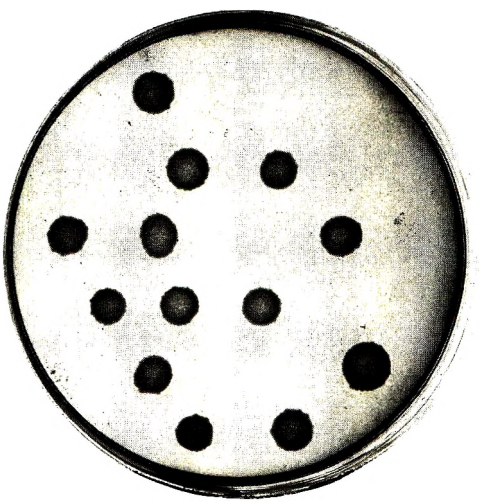

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EXPLANATION OF PLATES

PLATE 1

Resistance typing by strip-diffusion method. The central blotting-paper strip is impregnated with ampicillin Cultures (*a*)–(*d*) have the following MICs (μg./ml.): (*a*) 4 (sensitive control); (*b*) 32; (*c*) 16; (*d*) 500. (*e*) (*f*) (*g*) and (*h*) are duplicates of (*a*), (*b*), (*c*) and (*d*).

PLATE 2

Figs. 1 and 2. Sulphonamide resistance testing. In Fig. 1 the plate contains nutrient agar with 5% v/v lysed horse blood. In Fig. 2 the plate contains the same medium + 100 μg./ml. sulphathiazole. Cultures marked with crosses in Fig. 1 are sulphonamide-resistant. I = resistant control.

Figs. 3 and 4. Nalidixic acid resistance testing. In Fig. 3 the plate contains nutrient agar, and in Fig. 4 nutrient agar with 40 μg./ml. nalidixic acid. Cultures marked with crosses in Fig. 3 are nalidixic acid resistant. I = resistant control.

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