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The hygiene and marketing of fresh cream as assessed by the methylene blue test

A REPORT BY A WORKING PARTY* TO THE DIRECTOR OF THE PUBLIC HEALTH LABORATORY SERVICE

WITH A NOTE BY HILARY MOGFORD

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(*Received* 22 *July* 1970)

SUMMARY

The hygiene and marketing of fresh cream in England and Wales was investigated by a working party of the Public Health Laboratory Service (PHLS) between 1 October 1968 and 31 July 1969. Thirty-one cream-producing dairies were visited and observations made in the light of the Code of Practice published by the Milk and Milk Products Technical Advisory Committee of the Ministry of Agriculture, Fisheries and Food, and the Scottish Home and Health Department. Suggestions are made in this report to strengthen the code.

A total of 5184 samples of fresh cream comprising 4385 heat-treated, 282 clotted and 517 untreated were examined. Details of production, age of the cream, distance and mode of distribution along the retail chain were available for most samples, and this history was, as far as possible, related to the laboratory findings. In the laboratory the samples were examined by the methylene blue test, colony count, coliform and *Escherichia coli* I test and for pathogens. *Staphylococcus aureus* was grown from 59 out of 3417 samples of cream; 54 of these were from untreated cream. Phage typing indicated that a proportion of these strains were of animal, presumably bovine, origin. Other human pathogens isolated included one each of *Salmonella typhimurium*, *Brucella abortus*, *E. coli* type O126 and *Clostridium welchii*.

It appeared that heat-treated cream was much better, bacteriologically, than untreated; and that large dairies, in general, had better premises, more hygienic methods of preparation and the advantages of mechanical filling and capping of cartons; thus they offered the consumer a better product, bacteriologically, than some of the smaller dairies.

A statistical analysis of the results of the tests used showed the methylene blue

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ห้องสมุด กรมวิทยาศาสตร์

test, in spite of some anomalies, to be of more use as a screening test than any of the others. As this test is cheap and easy to carry out, the working party thought that it should be the test of choice. In view of the known anomalies, however, the working party recommends that the test should remain a screening or advisory test and have no penal function. Results should be reported as follows: cream not decolorizing the dye in 4 hr. at 37° C. (± 0·5°) after overnight incubation at 20° C. (± 0·5°) would be accepted as satisfactory; between ½ and 4 hr. at 37° C. (± 0·5°) as fairly satisfactory; but if the dye were decolorized at the end of the overnight incubation the sample would be provisionally classified as 'unsatisfactory; requires further investigation'. A repeat sample would then be examined. If samples were repeatedly unsatisfactory, consultations would be arranged between the local authority, the laboratory and the dairy, with perhaps inspection of the dairy premises and retail storage conditions and further, more searching tests on samples made in an effort to eradicate production faults.

INTRODUCTION

In England and Wales the regulations for the sale of milk do not wholly apply to cream. Milk is so defined as to include cream in the Milk and Dairies (General) Regulations 1959, which deal with the hygienic preparation of milk, but not in the Milk (Special Designation) Regulations 1963 which deal with licensing and statutory tests. Although cream must therefore be produced under hygienic conditions, a licence to produce cream is not required nor are there any laboratory standards to which cream must conform.

Despite the few incidents of food poisoning associated with cream (Cockburn & Simpson, 1954; Cockburn & Vernon, 1955, 1960; Vernon, 1964, 1967, 1969), the lack of laboratory tests, the increase in the volume of cream consumed as fresh cream (300 million cartons in 1968) and in cream cakes, and the known potential of cream as a medium for bacterial growth have caused disquiet to many bacteriologists.

A working party of the Public Health Laboratory Service (Report, 1958) examined fresh cream and found that, with the exception of creams pasteurized in the bottle, most creams examined were of poor bacteriological quality and were associated with a high degree of post-pasteurization contamination. Colenso, Court & Henderson (1966) confirmed these findings, showing that no less than 137 out of 575 samples had counts in excess of a million colonies per ml. As approximately 95 % of the 575 samples in this series had been heat-treated, the evidence for post-pasteurization contamination was conclusive. Many similar studies have been reported (Barrow & Miller, 1967; Barrow, Miller, Johnson & Hingston, 1968; Hutchison, Barrow, Henderson & Wright, 1968; Gerken, Coleman & Winner, 1968.) In these investigations good correlation was obtained between the time of reduction of methylene blue and the presence of coliform organisms. There were, however, anomalies, and for that reason the trade has preferred to examine cream by total counts and by tests for coliforms. Davis (1969) also suggested this.

A code of Hygienic Practice for Cream (1967) stresses that cream for human consumption should be made safe by heat treatment and that precautions for the

avoidance of contamination after treatment should be taken, but makes no reference to laboratory methods of determining the adequacy of heat treatment or to the avoidance of post-pasteurization contamination.

The effect of these publications coupled with the considerable increase in the consumption of cream in this country caused disquiet concerning the hygiene of this commodity. The PHLS therefore convened a Working Party whose terms of reference form the title of this paper and from whose report the substance of this paper is drawn. Laboratories participated from all over England, Wales and Northern Ireland, and between October 1968 and July 1969 a total of 5184 cream specimens were examined.

MATERIAL AND METHODS

When samples were collected the name and address of the producer was recorded together with the date of manufacture (if known), the date of receipt by the retailer, the type of container, and whether it bore a date of manufacture or code mark of any kind. The name and address of the retailer was also recorded, together with the method of conveyance from producer to retailer and the approximate length in road miles of the retail chain.

Members of the working party visited cream manufacturing dairies in their area to study the hygiene of the production methods employed and to inquire into the observance of the Code of Practice.

A total of 4385 heat-treated, 517 untreated and 282 clotted cream samples were examined. These were gathered over a wide area of England, Wales and Northern Ireland. Untreated cream formed a surprisingly large part (10 %) of the total amount of cream examined, with the highest proportion in the north-east of England. Clotted cream was almost entirely sampled by one laboratory in Truro.

The methylene blue reduction test, total count and coliform count were carried out as described in Report (1958).

The classification used for the identification of coliform organisms was that recommended by the *Coli–aerogenes* Subcommittee of the Society for Applied Bacteriology (Report, 1956).

For the purposes of the investigation, anomalous results (Jenkins & Henderson, 1969) were defined as decolorization of methylene blue immediately, with a colony count of less than 10^4 colonies/ml., and failure to decolorize methylene blue in 4 hr. with a colony count of more than 10^5 colonies/ml.

Samples were examined for salmonellas, brucellas, *Staphylococcus aureus* and other pathogens by the methods in use in each laboratory. Some laboratories undertook to carry out phosphatase tests on whole creams and creams diluted 1/10. A number of laboratories in addition carried out colony counts at 4, 20–22, 30 and 35° C. in addition to the customary 36 ± 1° C.

RESULTS

Table 1 shows the results of examination of 4385 heat-treated, 282 clotted and 517 untreated creams by the methylene blue test, colony counts, and presence in 0·1 ml of coliform organisms or *E. coli* I.

In general, samples shown to be satisfactory by one test were also satisfactory by the other tests, and vice versa. Thus, few samples graded as satisfactory by the methylene blue test contained coliforms or *E. coli* I in 0·1 ml. and few gave colony counts exceeding 10^5/ml. Many samples graded as unsatisfactory by the methylene blue test contained coliforms or *E. coli* I in 0·1 ml. and many such samples gave colony counts exceeding 10^5/ml.

Table 1. *Comparison of the methylene blue test, the coliform test, and colony counts on* 4385 *samples of heat-treated,* 282 *samples of clotted, and* 517 *samples of untreated cream*

(Figures in parentheses are percentages.)

	No.	Time (hr.) to decolorize methylene blue, and category according to the methylene blue test: >4, satisfactory	½–4, fairly satisfactory	0, unsatisfactory	Total, all categories
Heat-treated	4385	2283 (52)	936 (21)	1166 (27)	—
Clotted	282	185 (66)	70 (25)	27 (10)	—
Untreated	517	93 (18)	222 (42)	202 (39)	—
		Coliforms in 0·1 ml.			
Heat-treated	4385	160 (7)	319 (34)	762 (65)	1241 (28)
Clotted	282	32 (17)	33 (47)	16 (59)	81 (29)
Untreated	517	17 (18)	111 (50)	174 (86)	302 (58)
		Escherichia coli I in 0·1 ml.			
Heat-treated	4385	13 (<1)	62 (7)	136 (12)	211 (5)
Clotted	282	21 (11)	28 (40)	9 (33)	58 (20)
Untreated	517	5 (5)	51 (23)	93 (46)	149 (29)
		Colony counts* per ml. $\leqslant 10^3$			
Heat-treated	4385	1739 (76)	329 (35)	74 (6)	2142 (49)
Clotted	282	131 (71)	27 (39)	7 (26)	165 (59)
Untreated	517	40 (43)	31 (14)	5 (3)	76 (15)
		Colony counts* per ml. $> 10^5$			
Heat-treated	4385	81 (4)	177 (19)	734 (63)	992 (23)
Clotted	282	22 (12)	15 (21)	13 (48)	50 (18)
Untreated	517	5 (5)	70 (32)	153 (76)	228 (44)

* Colony counts at $36° \pm 1°$ C.

This general finding applied to heat-treated, clotted and untreated creams. *E. coli* I was, however, found in more samples of clotted cream (20 %) than of heat-treated cream (5 %).

Untreated creams were clearly inferior in bacteriological quality to heat-treated and clotted creams. Fewer samples had colony counts of less than 10^3/ml., and more contained coliforms and *E. coli* I in 0·1 ml.

Marketing

Tables 2–4 illustrate aspects of marketing and the effects of various conditions upon colony counts and coliform content. In Table 2 the results are grouped according to the season of the year. Colony counts, the coliform content and percentage of samples containing *E. coli* I of heat-treated and untreated cream rose as the mean monthly atmospheric temperature rose from its lowest level of 1·1° C. in February to its highest of 17·5° C. in July.

Table 2. *Fresh cream samples: total colony counts, coliform counts and* Escherichia coli I *counts with seasonal differences*

(Figures in parentheses are percentages.)

	October–March inclusive			April–July inclusive		
	Heat-treated	Un-treated	clotted	Heat-treated	Un-treated	clotted
Total ...	2268	228	215	2117	289	67
With coliforms in 0·1 ml.	560 (25)	114 (50)	60 (28)	703 (33)	184 (64)	21 (31)
With *E. coli* I in 0·1 ml.	81 (4)	57 (25)	42 (20)	130 (6)	92 (32)	14 (21)
With colony counts* of:						
$\leqslant 10^3$	1227 (54)	33 (15)	124 (58)	915 (43)	43 (15)	41 (61)
$> 10^3$–10^5	607 (27)	110 (48)	51 (24)	644 (30)	103 (36)	16 (24)
$> 10^5$	434 (19)	85 (37)	40 (19)	558 (26)	143 (50)	10 (15)

* Counts/ml. at $36 \pm 1°$ C.

Since organisms multiply more quickly in warm weather than in cold, coliforms perhaps present in too small numbers or not multiplying fast enough to be detected in the winter readily show their presence in the warm months. Although in the tables the highest counts are recorded as 10^5, counts as high as 4×10^7 to 2×10^9/ml. were not infrequent expecially in untreated samples.

The results of counts carried out at different temperatures are not recorded in the tables. Colony counts at 4°, 20–22° and 30° C. were on many occasions considerably higher than those recorded in the same samples incubated at $36 \pm 1°$ C. This was noted in particular when the predominant flora consisted of *Pseudomonas* spp. This is doubtless the explanation of the anomalous result, occasionally experienced, that some samples with colony counts of 10^3 fall into the class 'unsatisfactory' or 'fail' by the methylene blue test. Counts from such samples after incubation at 4° or 22° C. were sometimes surprisingly high.

Table 3 relates colony counts to the day of sampling. Counts in winter did not show much change until the fifth day and after. In summer the percentage of samples with counts of more than 10^5/ml. rose steadily from the day of manufacture.

Table 4 deals with the effect on the colony count of the type of carriage, distance carried and manner of storage for retailing. Unrefrigerated vehicles, e.g. milk floats, were in general use on milk rounds. Carriage of cream over long distances

was usually in refrigerated transport. Some creams sent by rail were enclosed in insulated or cooled boxes; others were not, even on long journeys. In general, colony counts were higher in creams carried in unrefrigerated vehicles.

Table 3. *Heat-treated cream: possible effect of age on colony counts*

	Day after manufacture when sample taken					
	0	1	2	3	4	⩾5
		November–March				
No. of samples	93	322	279	195	136	195
% showing colony counts* of						
⩽ 10^3	53	63	53	53	51	44
> 10^3–10^5	29	21	26	29	30	25
> 10^5	18	16	21	18	19	31
		April–July				
No. of samples	77	365	338	199	125	164
% showing colony counts* of						
⩽ 10^3	63	44	37	41	38	27
> 10^3–10^5	30	32	37	28	30	27
> 10^5	8	24	26	31	32	46

* At 36 ± 1° C.

Table 4. *Marketing of heat-treated cream: possible effects on colony counts of type of carriage, distance carried, and type of retail storage*

(Figures in parentheses are percentages.)

	Winter (Nov.–Mar.), no. of samples			Summer (Apr.–June), no. of samples		
	Total	With colony counts/ml.		Total	With colony counts/ml.	
		⩽ 10^3	> 10^5		⩽ 10^3	> 10^5
Type of vehicle						
Refrigerated	577	358 (62)	94 (16)	597	255 (43)	166 (28)
Unrefrigerated	460	218 (47)	114 (28)	482	171 (36)	158 (33)
Insulated	23	13 (57)	5 (22)	29	15 (52)	8 (28)
Distance transported (miles)						
⩽ 50	810	427 (53)	178 (22)	826	323 (39)	258 (31)
51– > 200	245	162 (66)	27 (11)	291	119 (41)	74 (25)
Type of storage						
Cooled	1002	565 (56)	193 (19)	960	408 (43)	269 (28)
Uncooled	63	24 (38)	22 (35)	149	34 (23)	63 (42)

Some creams travelled distances of more than 200 miles. Winter samples travelling 50–200 miles yielded lower colony counts than those travelling much shorter distances, but in summer this difference was not so obvious. The explanation probably is that cream sent long distances is usually produced by the larger firms. The production methods of the larger firms are generally better than those of small

dairies with local distribution. In addition, cream from larger firms is usually sent in refrigerated vehicles.

In retail shops most samples had been cooled in some manner or other. Most common was the 'cool display' in which cool air expelled upwards from a refrigerator circulated round a series of open shelves above the refrigerator. A large proportion of samples were kept simply in domestic refrigerators. In the larger dairies cream was kept in a 'walk-in' cold room. Little difference was seen in the colony counts of samples stored under these differing conditions. Rarely retail samples were found uncooled on open shelves, counters or market stalls, but such samples formed only a small percentage of samples examined. Both in winter and

Table 5. *Marketing of heat-treated creams: effect of storage at refrigerator temperature at source*

(Figures in parentheses are percentages.)

Days after manufacture	No. examined	No. of samples with: Colony counts of $\leqslant 10^3$	$> 10^3 - 10^5$	$> 10^5$	Coliforms in 0·1 ml.	*E. coli* I in 0·1 ml.
0	118	58 (49)	45 (38)	15 (13)	44 (37)	8 (7)
1	98	37 (38)	36 (37)	25 (26)	42 (43)	7 (7)
2	32	10 (31)	18 (56)	4 (13)	15 (47)	3 (9)
3	40	8 (20)	17 (43)	15 (38)	15 (63)	3 (8)
4	32	6 (19)	15 (47)	11 (34)	15 (47)	0
$\geqslant 5$	25	10 (40)	9 (36)	6 (24)	14 (56)	0

Table 6. *Marketing of untreated cream: effect of age on colony counts and coliform content*

(Figures in parentheses are percentages.)

Days after manufacture	No. examined	No. of samples with: Colony counts of $\leqslant 10^3$	$> 10^3 – 10^5$	$> 10^5$	Coliforms in 0·1 ml.	*E. coli* I in 0·1 ml.
			Winter			
0	28	1 (4)	16 (57)	11 (39)	14 (50)	10 (36)
1	78	12 (15)	35 (45)	31 (40)	40 (51)	18 (23)
2	75	11 (15)	41 (55)	23 (31)	40 (53)	19 (25)
3	47	7 (15)	18 (38)	22 (47)	20 (43)	10 (21)
Total	228	31 (14)	110 (48)	87 (38)	114 (50)	57 (25)
			Summer			
0	39	1 (3)	17 (44)	21 (54)	34 (87)	24 (62)
1	123	16 (13)	50 (41)	57 (46)	76 (62)	34 (28)
2	77	15 (20)	20 (26)	42 (55)	50 (65)	22 (29)
3	50	7 (14)	6 (12)	37 (74)	37 (74)	18 (36)
Total	289	39 (14)	93 (32)	157 (54)	197 (68)	98 (34)

summer there were more cooled samples with counts below 10^3/ml. and fewer with counts exceeding 10^5/ml. than of uncooled samples.

Tables 5 and 6 show the effect of ageing on heat-treated and untreated creams. In general, the colony counts and proportion of heat-treated samples with coliforms or *E. coli* I in 0·1 ml. increased with age even when these creams were stored at refrigerator temperature.

Table 7. *Hygiene and marketing of cream: comparison of samples from large and small dairies*

(Figures in parentheses are percentages.)

	Large dairies*		Small dairies†	
	Winter	Summer	Winter	Summer
Total samples ...	1191	1071	1520	1402
No. of samples				
With coliforms in 0·1 ml.	197 (17)	318 (30)	537 (35)	590 (42)
With *E. coli* I in 0·1 ml.	28 (2)	51 (5)	152 (10)	186 (13)
With colony counts/ml of				
$\leqslant 10^3$	763 (64)	483 (45)	620 (41)	516 (37)
$> 10^3$–10^5	258 (22)	318 (30)	510 (34)	455 (32)
$> 10^5$	170 (14)	270 (25)	390 (26)	431 (31)

* Large dairies with good equipment, mechanical filling, wide distribution in refrigerated vehicles.

† Small dairies using hand filling and local distribution in unrefrigerated vehicles.

Exact information about the age of untreated cream was difficult to obtain. It appears that the distribution of untreated cream is mainly local, and it was unusual to find untreated samples distributed more than 25 miles from where they were made. Usually they were carried in unrefrigerated vehicles and sold within 3–4 days after production. Colony counts were in general higher than those in heat-treated samples.

Table 7 compares the results of samples produced by large and small dairies. Those from large dairies were in general of better quality as judged by all tests than those from small dairies.

Bacterial multiplication in cream in the dairy and in the laboratory

In one laboratory bacterial counts were compared on 86 samples of heat-treated cream, some being stored at 4° C. in their original containers, others in sterile jars. A tenfold or greater increase was shown to take place in 73 % and coliforms originally present in 25 % were present in 57 % by the tenth day and were often accompanied by *E. coli* I not detected in the original specimen. There was no significant difference between the results of samples in the producers' containers and in sterile jars.

In other laboratories heat-treated cream was collected in sterile containers and examined in parallel after storage in the producer's cold room and in the laboratory refrigerator. Some 33 samples showed similar tenfold rises in bacterial counts over a period of 3–5 days. One specimen was split for storage at 4° C. in the laboratory, in the cold room in the dairy and on the milk float. Each portion showed a 5-day count of 4×10^5 colonies/g. due to *Pseudomonas fluorescens*.

Other workers observed a similar increase in counts over a 5-day period, coliforms and *E. coli*. I too few in number to be detected originally appearing at the end of this time even when stored at 4° C. In some instances counts rose from a few thousand to an excess of 12×10^5/ml.

New cream cartons taken at random showed no evidence of serious bacterial contamination.

Visits to cream-making dairies

Thirty-one cream-making dairies were visited. Nine (small) dairies made less than 100 gal. of cream a week, sixteen (medium) made an average of 100–1000 gal. per week and six (large) an average of over 1000 gal. per week. Christmas, Easter and the soft fruit season increased production, causing some dairies to buy in cream and market it under their own name.

Not all dairies tested the milk for keeping quality on arrival and three stored it overnight at room temperature.

Not all methods of heat-treatment of cream were satisfactory, for in one medium-sized dairy the cream, in a churn, was immersed in a bath of hot water. In two other dairies, one medium and the other large, flash pasteurization was used without accurate control of time or temperature.

In one establishment it proved difficult to divert pasteurized milk to the separator without the milk flowing over the hands of the operator.

Temperatures of separation showed wide variation, from the small dairy which separated milk still warm from the cow to the majority who separated between 35–50° C. The time taken during separation varied enormously but all producers then cooled and stored for 24 hr. to allow the cream to 'age'. Afterwards cartons were filled by jug or ladle and the lids applied by hand. All the large dairies, but only half of the medium-sized dairies, had mechanical fillers.

Code marks were not universally used and there seemed to be some reluctance to indicate that cream had been heat-treated. In addition to the sale of cartoned cream, some was sold in cans for subsequent redistribution into cartons in retail shops. This seems a most undesirable feature.

Pathogens

Staphylococcus aureus was isolated from 59 out of 3417 samples, 54 from untreated and 5 from heat-treated creams. Of 41 phage-typed, 5 were group I, 3 group III and 3 group IV; the remainder were mixed or untypable. This suggests that the strains were not all of human origin and some could be of animal, possibly bovine origin. Other pathogens isolated included *Salmonella typhimurium*, *Brucella abortus* type 1, *Escherichia coli* O126, all from untreated samples, and *Cl. welchii* from a treated sample.

Phosphatase testing

In Canada fresh cream must pass the phosphatase test, in Northern Ireland virtually all fresh cream is pasteurized and in Sweden fresh cream must be pasteurized. In this survey 90 % of the samples had been heat-treated and the phosphatase test was passed by 2649 out of 2957 of the heat-treated and clotted creams. Sixty-nine (2 %) showed readings between 10 and 18 μg. of para-nitrophenol per ml. of cream. Two hundred (7 %) failed the test in excess of 42 μg. The role of reactivation of phosphatase was not studied. Barrow *et al.* (1968) have shown that failures in this test have sometimes led to the demonstration of inadequate heat-treatment, but with a few exceptions heat-treatment apparently was being carried out according to the recommendations of the Code of Practice.

A bacteriological standard for cream

Standards in force in other countries include:

(1) Northern Ireland: pasteurized fresh cream, at source and within 24 hr. of manufacture, must be free of coliforms in 1 g.; farm-bottled, untreated cream, must have a count of less than 50,000 colonies/g.

(2) Canada: retail fresh cream must pass the phosphatase test, be free of coliforms and have less than 50,000 organisms/ml.

(3) Sweden: fresh cream must be pasteurized, have a count of less than 100,000 organisms/ml., a coliform count not exceeding 10/ml. and an aerobic spore count of less than 100/ml.

Table 5 shows that of 118 samples of heat-treated cream refrigerated at source and examined within 24 hr. of manufacture, 37 % would have been unacceptable under Northern Ireland standards. In fact 25 % of all heat-treated creams yielded coliform organisms. Additionally, cream in storage even at refrigeration temperatures allowed coliforms and salmonellas (Colenso *et al.* 1966) to multiply.

Colony counts were also considered. In this survey 37 % of untreated creams in winter and 50 % in summer had counts exceeding 10^5 colonies/ml. (Table 2). Of pasteurized creams 19 % in winter and 26 % of summer samples had counts in excess of 10^5/ml. All these would have failed the Canadian requirements. The Swedish regulations would have permitted a higher percentage of passes.

The application of a test for *E. coli* I shows a similar trend (Table 1). Of 5184 samples, 5 % of heat-treated, 20 % of clotted, and 29 % of untreated would have been rejected. On the other hand (Table 8), acceptance of samples free of *E. coli* I would have permitted 25 % of creams proved unsatisfactory by the methylene blue test to be marketed and an almost similar number (23 %) of those with colony counts in excess of 10^5/ml. Because of these observations the Working Party did not feel able to recommend any one of these three tests as suitable in itself for the statutory control of bacteriological quality of cream in the United Kingdom. For this reason it reconsidered the use of the methylene blue test as a clearance or screening test.

The results from all laboratories applying the methylene blue test are shown in Table 1, where it can be seen that 73 % of all heat-treated creams are acceptable.

Samples classed as satisfactory by the methylene blue test contain only 7 % of samples with coliforms present and 4 % with counts of over 10^5/ml. On the other hand, unsatisfactory samples contain a much higher percentage with coliforms and colony counts in excess of 10^5/ml. The test would fail 27 % of heat-treated creams, the coliform test 28 % and the colony count 23 %.

Table 8. *Comparison of results of* Escherichia coli I *test with methylene blue reduction test, presence of coliforms, and colony counts*

(Figures in parentheses are percentages.)

	E. coli I in 0·1 ml.	
	Absent	Present
Total samples ...	4753	431
Methylene blue reduced in (hr.)		
>4	2442 (52)	42 (10)
$\frac{1}{2}$–4	1105 (23)	144 (34)
0	1206 (25)	245 (57)
No. with coliforms in 0·1 ml.	1252 (26)	431 (100)
No. with colony counts of		
$\leqslant 10^3$	2241 (47)	32 (7)
$>10^3$–10^5	1441 (30)	126 (29)
$>10^5$	1071 (23)	273 (63)

Examination of the anomalies showed that where creams with low colony counts at 37° C. failed the methylene blue test, incubation at temperatures of 4° or 20° C. often revealed high colony counts. The converse anomaly where creams with high colony counts were satisfactory or fairly satisfactory with the methylene blue test was not explained. Nevertheless, the methylene blue test is a quick and easy test to perform and, as it bears a good correlation to other tests, the Working Party was of the opinion that it could serve excellently as a screening or advisory test provided the history of the specimen was known. Samples repeatedly found to be unsatisfactory would require further investigations with an inspection of dairy premises and retail storage conditions in an effort to eradicate production faults. In short, the methylene blue test is a good advisory test but it may not be used without further inquiry either to reject samples of cream or penalize a particular producer.

Code of practice

It appeared to the working party that dairy managers were in general putting into effect the recommendations in the Code of Hygienic Practice for the Preparation of Cream, issued in 1967 by the Ministry of Agriculture, Fisheries and Food, and the Scottish Home and Health Department. Only in one or two dairies was it clear that only lip service was paid to the code.

Suggestions for amendment of the Code were made as follows:

(1) The universal introduction of coding on cartons and bottles which could be clearly understood by traders and Public Health Inspectors.

(2) The application of a screening test such as the methylene blue test as a pointer to unsatisfactory practice. The test to lead to further investigations if the results were repeatedly unsatisfactory.

(3) The elaboration of section IV of the code to include recommendations on the structure and texture of equipment and utensils together with detail on cleansing measures. Section VII should also be strengthened and the use of paper towels and the care of the hands should be mentioned. Clearer instructions should be given for personnel with injuries. The present recommendation to use waterproof dressings could be interpreted as an invitation to continue work whilst suffering from a septic lesion.

(4) In the final paragraph in the code attention is drawn to the fact that the code has no statutory force. Further, the paragraph contains a number of apologetic statements destroying the whole spirit of the code. This paragraph should be reworded much more firmly and be followed by an exhortation to conform to the recommendations of the code.

(5) The wording of much of the Code could be more definite. Even the use of the words 'pure', 'sweet', 'clean' and 'marketable' are open to objection and might be replaced by wholesome. It is doubtful if the word 'pure' can be applied to such a complex mixture as cream.

DISCUSSION

The working party has confirmed that bacterial counts on retail cream are frequently high and that in this respect unheated is worse than heated cream. Some cream is prepared under unhygienic conditions.

Amongst the potentially pathogenic organisms which were isolated, staphylococci, possibly of bovine origin, were largely from untreated cream. Although no ill effects were reported from the 59 samples from which *Staphylococcus aureus* was isolated, food-poisoning outbreaks have occurred due to contamination of cream or cream products with this organism. Other organisms isolated from unheated specimens included *Salmonella typhimurium*, *Brucella abortus* and *Escherichia coli*, thus confirming the importance of heat-treatment. Nevertheless cream producers should be aware of the dangers of post-pasteurization contamination. This danger is particularly important where small shopkeepers, having no connexion with dairying, buy cream in 1 gal. cans and carton it for sale. In one instance it was shown that this process introduced *Staphylococcus aureus* into the sample. Officers of local authorities can take action in such instances under the 1959 Milk and Dairy (General) Regulations but there is strong demand from Public Health Inspectors for cream to be included in the 1963 Milk (Special Designation) Regulations. So far as hygiene of premises and production is concerned, the 1959 Regulations are quite strong but are not sufficiently used.

The larger dairies have many advantages, including good supervision and equipment, suitable premises, mechanical filling and capping of containers. Additionally, their large output necessitates distribution over a wide area but this is done in modern refrigerated vehicles. The small producers on the other hand often prepared cream under unsuitable conditions and filled and capped the cartons by hand.

Very often the smaller producer handled unheated cream and this was distributed locally, rarely exceeding a radius of 25 miles. Although the results reflected these differences, as may be seen in Table 7, not all small producers made poor cream, for some small family businesses were excellently run and produced cream of a consistently high quality.

Members of the working-party would like to invite the attention of cream producers to a particularly undesirable feature of cream, namely the presence of coliform organisms in a large number of heat-treated samples that have apparently been handled under reasonably good conditions. Loss of keeping quality soon after production might well be the only apparent result of the presence of large numbers of coliforms, but the presence of these organisms in heat-treated samples must surely indicate inadequate heat-treatment, unsterile equipment, contamination from the environment, unsatisfactory conditions of storage and distribution, or a combination of these. The working party thinks this practical problem is one to which the trade could address itself; its solution would be of obvious benefit to the trade and the consumer.

The working-party is of the opinion that the best hope of improvement in cream production lies in a Code of Practice suitably strengthened by the recommendations contained in this report.

Members of the working party are indebted to Mrs Ruth Homes of the Public Health Laboratory, Worcester, for her arduous work in collating and analysing the results, and all Public Health Inspectors, Sampling Officers, laboratory technicians and others who helped in the survey.

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Statistical comparison between results from the methylene blue test and three other tests of the bacteriological quality of cream samples

By HILARY MOGFORD

The three tests of colony count, coliforms in 0·1 ml. and *Escherichia coli* I in 0·1 ml. were each used in turn as a reference test. The findings of the methylene blue test were then compared with the reference test. The criteria for failing the four tests were 'unsatisfactory' to the methylene blue test, a colony count of more than 100,000 per ml. at 36° C ($\pm$ 1°), coliforms found to be present in 0·1 ml., and *E. coli* I found in 0·1 ml. The tables divide up the three lots of cream samples according to whether or not they failed the methylene blue and reference tests (Tables 9–11).

The analysis aims at seeing how well the methylene blue test does compared with the other three. There are two ways in which the test under observation can fail to agree with the reference test. It can be insensitive and not fail all the samples failed by the reference test, and it can be non-specific and fail many of the samples which it should have passed. The following statistical indices were evaluated for each of the nine test pairs.

a = sensitivity = proportion of all samples failing the reference test which are failed by the methylene blue test.

b = specificity = proportion of all samples passing the reference test which are passed by the methylene blue test.

J = Youden's Index (Youden, 1950) = combined estimate of sensitivity and specificity. It lies between zero, if the test is doing no better than random, and 1, if the test is producing exactly the same results as the reference test.

The standard error of J is also given.

Throughout the tables the methylene blue test was more specific than sensitive, especially with the clotted-cream samples. This implies that it made a higher rate

of mistakes in passing failures to the reference test than in failing samples which had passed the reference test.

The value of J, which assesses the overall agreement, shows up as highest between methylene blue test compared with the colony count and lowest with the

Table 9. *Heat-treated cream: comparison of methylene blue test with other tests*

Methylene blue test	No. of samples	Colony count		Coliforms in 0·1 ml.		*E. coli* I in 0·1 ml.	
		F	P	F	P	F	P
Failed	1166	734	432	762	404	136	1030
Passed	3219	258	2961	479	2740	75	3144
Total	4385	992	3393	1241	3144	211	4174
		$a = 0{\cdot}74$		$a = 0{\cdot}61$		$a = 0{\cdot}64$	
		$b = 0{\cdot}87$		$b = 0{\cdot}87$		$b = 0{\cdot}75$	
		$J = 0{\cdot}61$		$J = 0{\cdot}49$		$J = 0{\cdot}40$	
		S.E.$(J) = 0{\cdot}02$		S.E. $(J) = 0{\cdot}01$		S.E. $(J) = 0{\cdot}03$	

Table 10. *Clotted cream: comparison of methylene blue test with other tests*

Methylene blue test	No. of samples	Colony count		Coliforms in 0·1 ml.		*E. coli.* I in 0·1 ml.	
		F	P	F	P	F	P
Failed	27	13	14	16	11	9	18
Passed	255	37	218	65	190	47	208
Total	282	50	232	81	201	56	226
		$a = 0{\cdot}26$		$a = 0{\cdot}20$		$a = 0{\cdot}16$	
		$b = 0{\cdot}94$		$b = 0{\cdot}95$		$b = 0{\cdot}92$	
		$J = 0{\cdot}20$		$J = 0{\cdot}14$		$J = 0{\cdot}08$	
		S.E. $(J) = 0{\cdot}06$		S.E. $(J) = 0{\cdot}05$		S.E. $(J) = 0{\cdot}05$	

Table 11. *Untreated cream: comparison of methylene blue test with other tests*

Methylene blue test	No. of samples	Colony count		Coliforms in 0·1 ml.		*E. coli* I in 0·1 ml.	
		F	P	F	P	F	P
Failed	202	153	49	174	28	93	109
Passed	315	75	240	128	187	56	259
Total	517	228	289	302	215	149	368
		$a = 0{\cdot}67$		$a = 0{\cdot}58$		$a = 0{\cdot}62$	
		$b = 0{\cdot}83$		$b = 0{\cdot}87$		$b = 0{\cdot}70$	
		$J = 0{\cdot}50$		$J = 0{\cdot}45$		$J = 0{\cdot}33$	
		S.E. $(J) = 0{\cdot}04$		S.E. $(J) = 0{\cdot}04$		S.E. $(J) = 0{\cdot}05$	

E. coli I test. This held for all three types of cream sampled. Generally, the index is high for heat-treated samples but poor for clotted cream. In fact it does not differ significantly from zero for the methylene blue and *E. coli* I tests comparison.

Most cream samples encountered in laboratories are heat-treated and it is with

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this type that the methylene blue test proved to be the most promising as a potential screening test of bacteriological quality. It is likely to fail about three-quarters of the samples which would have colony counts of more than 100,000 per ml. and fail about one in eight of the samples with lower colony counts.

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Pyocine typing as an epidemiological marker in *Pseudomonas aeruginosa* mastitis in cattle

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SUMMARY

Pyocine typing was used for the characterization of 134 *Pseudomonas aeruginosa* strains isolated from bovine mastitis. The scheme of Gillies & Govan (1966) was adopted with some modifications, and the procedure gave 89·6 % typability. Pyocine type 1 strains were most commonly encountered and were followed in frequency by types 10 and 3. The introduction of two additional indicator strains allowed for division of these types into subtypes.

In spite of some limitations, discussed in the paper, the pyocine typing scheme proved to be useful in 'marking' *P. aeruginosa* strains and in following their association with bovine mastitis in various herds.

INTRODUCTION

Pseudomonas aeruginosa is widely distributed in nature and consequently the udder of the dairy cow may at times be exposed to this organism. Reports on sporadic cases of bovine mastitis due to this agent appeared in the early literature (Pickens, Welsh & Poelma, 1926; Cherrington & Gildow, 1931; Cone, 1939). However, more recently, attention has been drawn to the increasing incidence of *P. aeruginosa* mastitis and in some instances the disease was reported to be a major herd problem (Plastridge, 1958; Nurmi & Koiranen, 1967), characterized by recurrent infections (van Kruningen, 1963; Schalm, Lasmanis & Carroll, 1967). It was suggested (Mastitis Sub-Committee, 1965) that herd outbreaks might be attributed to the introduction for use in cowsheds of quaternary ammonium compounds to which *P. aeruginosa* proved to be resistant, to contaminated intramammary chemotherapeutic preparations and contaminated milking equipment (Tucker, 1950) and to contaminated water (Redaelli & Perini, 1960; Curtis, 1969).

During investigations of the control of bovine mastitis in Israel (Ziv, 1971) it was found that in several herds, in spite of efficient husbandry and sanitary milking practices, *P. aeruginosa* infected as many as 80 % of the cows' udders. This contributed to severe economic losses. In the course of an investigation aiming at tracing the source of infection, the organism was recovered from the skin of the teats and udders, from the milking equipment, from the floor, walls

and bedding in the milking sheds, from the drinking water in utensils but not from the main water supply.

It was of interest to follow the epidemiology of infections and to 'finger-print' the strains of *P. aeruginosa*. The pyocine typing scheme of Gillies & Govan (1966) was adopted with some modifications. The procedure is based on the ability of most *P. aeruginosa* strains to produce pyocines, which are specific antibiotic-like substances with a lethal activity, mainly restricted to other members of this species. Pyocine types are recognized by the patterns of growth inhibition of selected indicator strains.

The present communication records the pyocine typing of *P. aeruginosa* strains, mostly from cases of bovine mastitis, and a few from the cows' environment.

MATERIALS AND METHODS

Origin of Pseudomonas aeruginosa *strains*

Procedures used for the milk sampling and for the isolation and identification of *P. aeruginosa* have been described (Ziv, 1971). The organism was recovered from the secretions of 134 quarters (glands) of 76 cows in 26 dairy herds. In most herds *P. aeruginosa* was cultured from one to three cows only, but in each of four herds about 80 % were infected. Infection of a single quarter of the udder was the most common feature, but 22 cows were infected in two quarters, and nine cows in three or four quarters. In addition, 14 *P. aeruginosa* strains recovered from the previously infected quarters after an interval of 5 months were available for the study of the constancy of infections with particular pyocine types.

Pyocine typing

The procedure for pyocine typing was similar to that described by Gillies & Govan (1966), but we have introduced an apparatus for simultaneous streaking of indicator strains instead of individual application. Furthermore, to the original set of eight indicator strains, I_1 to I_8, received by the courtesy of Dr Gillies and Dr Govan, we have added two of our own indicator strains labelled I_A and I_B. The apparatus used consisted of two parts, one contained ten wells for the indicator strains, which were broth cultures grown for 4 hr. at 37° C., and the other part (named 'broomette') had ten prongs with rounded tapered ends. The prongs were inserted into the wells and then streaked across the plate at a right angle to the line of the original inoculum of the producer strain. The patterns of inhibition ef the indicator strains were recorded after 16 hr. incubation at 32° C.

Drug sensitivity tests

The procedure has been described previously (Ziv & Risenberg-Tirer, 1970). The minimal bactericidal concentrations (MBC) were determined using the following drugs: carbenicillin, polymyxin B, colistin, gentamycin, tetracycline, dihydrostreptomycin, kanamycin, neomycin and nalidixic acid.

RESULTS

Of 134 *P. aeruginosa* strains isolated from bovine udders and typed with the eight indicator strains of Gillies & Govan (1966), 120 (89·6 %) proved to be typable. As shown in Table 1, eight pyocine types were differentiated and type 1, represented by 88 strains, was most frequently encountered. Amongst the other strains, 15 were type 10, 6 were type 3, and the remaining types were 6, 2, 11, 7 and 31. The introduction of two additional indicator strains I_A and I_B provided a useful division of the most frequently encountered types 1, 10 and 3 into subtypes 1^{++}, 1^{+-}, 10^{++}, 10^{--}, 3^{++}, and 3^{+-}.

Table 1. *Distribution of pyocine types in* 134 Pseudomonas aeruginosa *strains isolated from bovine mastitis*

Pyocine		Strains		
Type	Subtype	Number		%
1	+ +	73	88	65·7
	+ −	15		
10	+ +	2	15	11·2
	− −	13		
3	+ +	3	6	4·5
	+ −	3		
6			4	3·0
2			3	2·2
11			2	1·5
7			1	0·7
31			1	0·7
Untypable			14	10·5

Table 2. *Distribution of pyocine types in* Pseudomonas aeruginosa *strains isolated from cows' udders in four herds, each with about* 80 % *incidence of infection*

Herd code	No. of infected quarters	Percentage of quarters infected with strains of pyocine type					
		1 + +	1 + −	10 − −	3 + −	11	Untypable
M	9	0	33	67	0	0	0
P	20	55	0	15	0	10	20
A-6	18	100	0	0	0	0	0
Z	15	60	20	0	20	0	0

A different pyocine type pattern was noted in *P. aeruginosa* strains from each of the four herds with the incidence of infections reaching about 80 % (Table 2). All strains isolated from cows in herd A-6 were pyocine type 1^{++}, and this was also the type of strains recovered from the skin of the udder and teats of these animals and from various fomites such as milking equipment and utensils used for this particular herd. Pyocine type 1 strains were predominant in herds P and Z but were not encountered in herd M, whereas in herds Z and M strains of subtype 1^{+-} were recorded. In herds M, P and Z the other strains were pyocine type 10^{--}, or

3^{+-}, or the rare type 11; 20 % of strains in herd P were found to be untypable. The above data, although limited in number, indicate that different pyocine type patterns may be found in *P. aeruginosa* strains from various dairy herds, and may serve as useful markers for epidemiological surveys.

Table 3. *The pyocine types of* Pseudomonas aeruginosa *strains isolated from 22 cows each infected in* 2 *quarters of the udder*

Herd code	Cow no.	Pyocine type found in the	
		First quarter	Second quarter
A	1	1++	1++
P	1	1++	1++
P	8	1++	Untypable
Q	2	1++	1++
G	1	1++	1++
L	2	1++	1++
L	4	1+−	10−−
M	2	1+−	10−−
M	7	1+−	10−−
A-1	1	1+−	10−−
A-2	1	1+−	1+−
Z	2	1++	3+−
Z	3	1++	1++
Z	5	1++	3+−
Z	7	1++	1++
A-6	2, 3, 4, 5 6, 8, 10	1++	1++

Table 4. *The pyocine types of* Pseudomonas aeruginosa *strains isolated from nine cows each infected in* 3 *or* 4 *quarters of the udder*

Herd code	Cow no.	Pyocine type found in various quarters			
		First	Second	Third	Fourth
P	5	UT	UT	10−−	
P	9	1++	11	11	1++
P	10	1++	1++		10−−
Q	1	10−−	1++	1++	
T	2		2	2	1++
O	1	1+−		1+−	1+−
Y	1	UT	UT	UT	UT
Y	2	1++	1++	1++	UT
Z	8	3+−	1+−	1+−	

UT = untypable strains.

A comparison was made between the pyocine types of *P. aeruginosa* strains isolated from 22 cows, each infected in two quarters. Table 3 shows that strains of the same pyocine type infected both quarters of 15 (68 %) cows. Further, a comparison was made between pyocine types of strains isolated from nine cows, each with multiple infection of three or four quarters. It was found (Table 4) that in every case, with the possible but unlikely exception of cow Y 1, at least two quarters

were infected with *P. aeruginosa* of the same pyocine type. It should be noted that infection of two quarters of the udder was recorded with the rare types 2 and 11. It seems, therefore, that a fair degree of uniformity was shown by the simultaneous presence of organisms of the same pyocine type in different quarters of the udder.

The availability of 14 *P. aeruginosa* isolates from udders of cows which were found to be infected with this species 5 months previously, provided the opportunity of assessing whether the same or different pyocine types appear on repeated examination. Table 5 shows that strains of the same pyocine type were encountered only in five cases.

Results of the drug sensitivity tests did not reveal any significant relationship between the patterns obtained and the various *P. aeruginosa* pyocine types.

Table 5. *Constancy of pyocine types in strains of* Pseudomonas aeruginosa *obtained after* 5 *months from the same quarters in the same cow*

		Pyocine type found	
Herd code	Cow no.	On first examination	After 5 months
M	2	10 − −	1 + −
O	1	1 + +	1 + +
P	1	1 + +	1 + +
P	1	1 + +	UT
P	2	1 + +	7
P	3	10 − −	1 + +
P	8	UT	1 + +
P	9	1 + +	1 + +
P	9	1 + +	1 + +
P	10	10 − −	UT
P	11	1 + +	1 + +
L	1	1 + +	UT
L	2	1 + +	UT
L	4	10 − −	1 + −

UT = untypable strains.

DISCUSSION

Bacteriophage typing and serotyping have been employed by various workers to differentiate strains of *P. aeruginosa*. However, these procedures require the preparation and maintenance of a collection of phages and diagnostic sera. The introduction of pyocine typing schemes (Darrell & Wahba, 1964; Gillies & Govan, 1966) provided simpler techniques for use in diagnostic laboratories and were adopted by various workers for epidemiological studies.

In the present survey the method of Gillies & Govan, applied to the pyocine typing of *P. aeruginosa* strains from bovine mastitis in Israel, gave 89·6 % of typable strains. No record has been found in the literature of this procedure being applied in surveys of bovine pseudomonas mastitis in other countries. Govan & Gillies (1969) gave the figure 7·6 % of untypable strains in an investigation of pyocine typing of clinical strains in Scotland, while J. R. Tagg and R. Mushin

(unpublished data) listed 8·6 % in their series from hospitals in Australia. Pyocine type 1 strains were most commonly encountered in the above three surveys, concerned with man and animal. In the present study strains of pyocine type 10 and 3 came next on the list of frequency of appearance, as compared with pyocine types 3 and 5, and 3 and 10 in the Scottish and Australian surveys, respectively.

The addition of two indicator strains I_A and I_B allowed the division of the more commonly occurring pyocine types 1, 10 and 3 into subtypes.

It is of interest to note the results of pyocine typing of material from other sources in Israel. Thus of 15 *P. aeruginosa* strains from laboratory mice, 12 were type 3^{+-} and three were untypable, while 35 strains from human sources were predominantly type 1^{++} (49 %), less frequently types 1^{+-} and 10^{++} and occasionally types 35, 6, 3^{+-} or untypable (G. Ziv, unpublished).

The examination of *P. aeruginosa* strains from two or more quarters of the same udder, and from the same quarters after an interval of 5 months, showed at times the presence of different pyocine types. The present results are comparable with those reported by Gillies & Govan (1966) and Govan & Gillies (1969) who found that in a series of *P. aeruginosa* strains from human cases, some belonged to different pyocine types from those originally isolated from the same site of the patient. Evidence pointed to the possibility of multiple infections rather than to instability of the pyocine types.

In conclusion, in spite of the occurrence of some untypable strains and the limited number of pyocine types, the scheme seems to be of practical value in characterizing *P. aeruginosa* strains associated with bovine mastitis.

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Pseudomonas aeruginosa in a Regional Burns Centre

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SUMMARY

The construction of a Regional Burns Centre in Pinderfields General Hospital, Wakefield, presented an opportunity to study *Pseudomonas aeruginosa* infection in patients with extensive burns. During the first year (Barclay & Dexter, 1968) a system of disinfection and bacteriological control created conditions permitting more detailed studies to be undertaken which resulted in a significant reduction of infection and cross-infection.

MATERIALS AND METHODS

Twelve-hour culture settle plates (Oxoid blood agar base No. 2) were placed in all parts of the burns unit each day with a view to assessing bacterial fall-out from the air for each locality. Colony counts and types of bacteria were studied in association with treatment and nursing procedures, and served as a guide to determine the most effective methods of controlling the bacteria by disinfection. Water supplies, drain systems, sluices, etc., were also examined and disinfection procedures were instituted as required. *Pseudomonas* strains grown from patients and their surroundings were pyocine typed (Darrell & Wahba, 1964; Gillies & Govan, 1966, Govan & Gillies, 1969) to establish identity or differences. The isolation of *Pseudomonas* in pure culture was by using 0·03 % Cetrimide in (Oxoid) blood agar base No. 2 and 0·02 % Centrimide in (Oxoid) Nutrient broth No. 2 (Shooter *et al.* 1966). Other routine medium was also used and disinfectant inactivators were included as required.

During the first year 'Idokyl', an iodophor, was the only disinfectant used, but during the subsequent years 'Tego' (MHG), an ampholytic surface-acting biocide disinfectant, was used. Both disinfectants were applied as aerosol mists (Barclay & Dexter, 1968), followed by a disinfectant surface wash; aerosols were never used as a substitute for washing. By this method bacteria were first precipitated on surfaces which were immediately washed, killing most microbial survivals.

RESULTS

Airborne Pseudomonas aeruginosa

Recovering of *Pseudomonas* from settle plates on the floor of infected patients' cubicles, treatment rooms and traffic zones indicated widespread distribution of this organism via the air as evidenced by pyocine typing results. It was also noted

that settle plates situated on window ledges several feet high consistently yielded about the same number of *Pseudomonas* colonies as settle plates on the floor, and it was considered that the air in treatment rooms might be continuously contaminated with *Pseudomonas*. In attempting to assess the incidence of air-borne *Pseudomonas* under more controlled conditions, simple structures were prepared permitting daily 7 hr. settle plates to be placed at intervals of 1 ft. between the floor and ceiling of treatment rooms (Barclay & Dexter, 1968). These were in addition to the routine 12 hr. settle plates on the floor. These structures were called 'gravitation trees' and several were used in the cubicles of infected and uninfected

Table 1. *Extract from records* (14-*day period only*) *of colony counts of* Pseudomonas *on cultures on one of the 'gravitation trees' in a cubicle occupied by an infected patient* (*this patient did not receive topical cream treatment*)

Height above floor (ft.)	Colony counts of *Pseudomonas* on day:													
	3	4	5	6	7	8	9	10	11	12	13	14	15	16
9	1	1	0	13	2	11	0	1	0	13	0	5	0	8
8	3	3	1	15	3	18	1	0	1	22	1	6	0	8
7	0	5	0	11	0	14	0	18	0	11	1	7	1	11
6	1	1	1	11	0	15	0	15	4	20	0	6	0	5
5	0	1	1	9	0	12	2	19	1	16	0	5	0	2
4	3	2	0	5	0	20	1	17	1	23	1	9	0	5
3	3	3	1	5	2	14	0	19	3	25	1	7	0	12
2	0	7	1	19	0	20	0	13	1	13	1	8	4	5
1	0	6	1	16	1	13	0	15	0	17	0	3	0	7
Floor	0	2	1	15	3	11	0	14	1	17	0	5	0	13

patients, traffic zones and other treatment rooms in the unit. The results clearly demonstrated that living *Pseudomonas* was randomly distributed in the air of rooms in which infected patients were being treated (Table 1). Persistence of *Pseudomonas* in the air during the more active daily periods and less active nightly periods continued throughout the infective phase of patients' wounds. Continuous shedding of *Pseudomonas* from wounds on bedding resulted in frequent eruptions of this organism into the air when patients moved, or during treatment and bed linen changes. It was found that the presence of *Pseudomonas* in the air originated from infected patients, whereas the remaining bacteria in the air had many other influencing factors. Effective control of bacteria in the air of infected patients' cubicles did not achieve control of *Pseudomonas* in the air, and there appeared to be no relationship between *Pseudomonas* and the remaining bacterial population.

In the Burns Centre positive-pressure filtered air was provided in the dressings-treatment room, and air extraction in the dirty utility room (Barclay and Dexter 1968). Heavy growths of *Pseudomonas* were recovered from settle plates in all the rooms of the dressing suite during treatment of infected patients, and *Pseudomonas*, of the same pyocine type as that carried by the patient receiving treatment, was also recovered from other parts of the burns unit remote from either the patient or the dressing suite. It was established beyond doubt that the positive-pressure ventilation system was responsible for the widespread airborne distribu-

tion of this organism. The positive pressure was eventually replaced by negative pressure with a positive-pressure air-lock at the entrance to the dressings suite. From this time, *Pseudomonas* was effectively contained within the dressings suite during treatment of infected patients.

Surface-borne Pseudomonas aeruginosa

Press-plate cultures (Oxoid blood agar base No. 2) were mainly used to recover *Pseudomonas* from surfaces, and recoveries were made from many parts of the environment, including gowns and overshoes worn by staff. These led to recoveries being often made at considerable distances from confirmed sources. *Pseudomonas* recoveries from surfaces in infected patients' cubicles rapidly diminished after discharge of patients. In moist conditions, however, where growth of the organism was possible, it could become established as resident and create additional sources of infection.

Table 2. *Disinfection and bacteriological control of 73 saline-bath treatments*

Organisms	No. of times the listed organisms were grown		
	Before bath	During bath	After disinfection
Ps. aeruginosa	1	44	2*
Proteus spp.	0	30	3
Staph. aureus	1	25	0
Coliforms	0	15	1
Staph. albus	5	0	1
Enterococci	1	11	0
B. anthracoides	0	1	0
Strep. pyogenes	0	0	0
Strep. viridans	0	0	0
Diphtheroids	0	0	0

* Untrained staff used incorrect disinfection procedure on these two occasions.

When an infected patient was given treatment in the saline bath, of which there was only one in the unit, the inner bath surface was found to become heavily contaminated with *Pseudomonas* and other organisms, and thus became a potential source of infection. This is shown in Table 2, which also shows the effects of disinfection of the bath with 'Tego' (MHG) after treatment of infected patients. A routine system of disinfection and bacteriological control was applied for each bath treatment. The inner bath surface was first wiped with undiluted 'Tego' and washed with water. After saline had been added to the bath, the patient was immersed using a hydraulic hoist, and on completion of treatment the patient was removed and the dirty water drained off. The bath was then two-thirds filled with a 1/100 dilution of Tego into which the hydraulic hoist was immersed for not less than 30 min.; the bath was then washed with water. Swabs for bacteriology were taken from the inner bath surface before the first application of undiluted Tego, after the dirty water had been drained from the bath, and on completion of disinfection.

Resident Pseudomonas aeruginosa.

Persistence of *Pseudomonas* in a growing state provides new sources of infection and may lead to successive patients being infected. Soap dishes, denture and receiving bowls, water in flower vases, sink cloths, cleaning utensils, sinks and bath overflows and particularly sink traps are typical examples, and when established over a long period the original sources of contamination can no longer be traced. With so many additional sources of infection available it becomes difficult to study the distribution of *Pseudomonas* or determine how patients become infected. When such a source of infection is revealed, the pyocine types of *Pseudomonas* found may be those from past or present patients, from patients in other parts of the hospital (transmission by staff), or from contaminated materials, including hands which have been washed in contaminated sinks. The efficiency of removing *Pseudomonas* from hands by washing after contact with infected patients was tested, and invariably resulted in living *Pseudomonas* being deposited in the sink bowls and sink traps, with the attendant risk of survivals on the hands being transmitted to other patients. Residue of hand washing under these conditions provides a continuous build-up of resident *Pseudomonas* and considerably increases the risk of other patients becoming infected. Considering the number of times each day that hands are washed after contact with infected patients, it is not surprising that infection of severe burn wounds, although undesirable, is accepted as inevitable. It has been established over a 4-year-period that regular disinfection is an effective method of preventing resident *Pseudomonas* from becoming established. Sink traps were dosed on alternate days with 1 fluid oz. of undiluted Tego, which was also used to wipe the inner surface of the sink bowls. Cleansing utensils were completely immersed in a 1/40 dilution of Tego when not in use. Flower vases with water were not permitted in the unit during the first 3 years. Within a few weeks of their introduction during the fourth year *Pseudomonas* was recovered in a growing state from a vase, but not before it was established that dirty water from the vase had been ejected into several sinks in the unit. Further enquiries revealed that this vase had been placed in different patients' cubicles over a period of time. Examination of the contaminated sinks and sink traps did not show any living *Pseudomonas* and further confirmed the effectiveness of disinfection. In all instances, the methods of disinfection were applied after bacteriological examination had proved them to be effective.

Control of cross-infection

Air borne *Pseudomonas* mainly originates in dry infected necrotic tissue broken down into minute *Pseudomonas*-laden particles which are frequently shed into the air. It seemed that if these particles were confined to the wounds of infected patients, airborne contamination would be considerably reduced. Anti-bacterial creams (Aserbine, Gentamycin, Sulfamylon, etc.) were liberally applied to wound areas, which resulted in a dramatic reduction of *Pseudomonas* being shed from the patients into the air (Table 3). It was realized, however, that although the creams were effective as bacterial suppressive agents, living *Pseudomonas* was still present

Table 3. *Extract from records* (14-*day period only*) *of colony counts of* Pseudomonas *on cultures on one of the 'gravitation trees' in a cubicle occupied by an infected patient* (*this patient received topical cream treatment*)

Height above floor (ft.)	Colony counts of *Pseudomonas* on day:													
	3	4	5	6	7	8	9	10	11	12	13	14	15	16
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	1	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	1	0	0	0	0	0	0	0	0	0	0	1	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Floor	0	1	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. *Pyocine typing* 1966: *chain-like infections with two types of* Pseudomonas aeruginosa *affecting* 23 *patients, suggestive of cross-infection*

Patient	Admitted	Pyocine types of *Pseudomonas aeruginosa*						
E.L.	Dec. 65	—	F	—	—	—	—	—
C.L.	Jan. 66	—	—	O3	—	—	—	—
D.E.	Feb. 66	—	—	—	B1C	—	—	—
I.B.	Feb. 66	B1A	—	—	—	—	—	—
N.E.	Feb. 66	—	—	—	B1C	—	—	—
S.M.	Mar. 66	—	—	—	B1C	—	—	—
A.F.	Mar. 66	—	—	—	B1C	—	—	—
W.I.	Apr. 66	—	—	—	—	B1B	—	—
G.O.	Apr. 66	—	—	—	B1C	—	—	—
S.I.	Apr. 66	—	—	—	B1C	—	—	—
B.E.	Apr. 66	—	—	—	B1C	—	—	—
M.I.	Apr. 66	—	—	—	—	B1B	—	—
W.R.	May 66	—	—	—	B1C	—	—	—
H.O.	June 66	—	—	—	—	B1B	—	—
W.I.A.	June 66	—	—	—	—	B1B	—	—
S.H.	July 66	—	—	—	—	B1B	—	—
C.R.	Aug. 66	—	—	—	—	B1B	—	—
W.I.	Sept. 66	—	—	—	—	—	N5	—
R.I.	Sept. 66	—	—	—	—	B1B	—	—
H.E.	Sept. 66	—	—	—	—	B1B	—	—
W.E.	Sept. 66	—	—	O3	—	—	—	—
W.H.	Oct. 66	—	—	—	—	B1B	—	—
H.O.L.	Oct. 66	—	—	—	—	B1B	—	—
B.A.	Nov. 66	—	—	—	—	B1B	—	—
S.E.	Nov. 66	—	—	—	—	B1B	—	—
L.O.	Nov. 66	—	—	—	—	B1B	—	—
H.E.	Nov. 66	—	—	—	—	—	—	NT
B.U.	Nov. 66	—	—	—	—	B1B	—	—
S.H.	Dec. 66	—	—	—	—	B1B	—	—

on infected wounds, and could be transmitted to other patients by direct contact.

Out of a total of 71 patients who were admitted into the unit during 1966, the wounds of 29 (41 %) were colonized with Pseudomonas, and 23 of these produced infections of two distinct pyocine types, which indicated that they were cross-infected by direct contact (Table 4).

Table 5. *Pyocine typing* 1967: *random scatter of pyocine types resulting from cross-infection counter measures*

Patient	Admitted	Pyocine types of *Pseudomonas aeruginosa*						
G.E.	Jan. 67	—	—	—	—	—	Q 22	—
H.U.	Jan. 67	—	—	—	—	—	—	O 25
W.E.	Jan. 67	—	—	—	—	—	—	O 25
B.R.	Feb. 67	—	—	—	—	—	—	O 25
M.I.	Feb. 67	—	—	—	B 1 B	—	—	—
F.I.	Feb. 67	—	—	—	—	—	Q 22	—
C.L.	Feb. 67	—	—	—	—	NT	—	—
H.O.	Apr. 67	—	—	—	B 1 B	—	—	—
S.I.	May 67	—	P 30	—	—	—	—	—
H.U.	May 67	—	—	—	—	NT	—	—
H.U.	June 67	B 1 A	—	—	—	—	—	—
A.P.	July 67	—	—	—	—	NT	—	—
C.O.	Aug. 67	—	—	—	—	NT	—	—
W.I.	Aug. 67	B 1 A	—	—	—	—	—	—
A.C.	Sept. 67	—	—	F	—	—	—	—
A.P.	Oct. 67	—	—	—	—	NT	—	—
T.H.	Nov. 67	—	—	—	—	NT	—	—
H.A.	Nov. 67	B 1 A	—	—	—	—	—	—
W.A.	Dec. 67	—	—	—	B 1 B	—	—	—
O.L.	Dec. 67	—	—	—	B 1 B	—	—	—

Elimination of cross-infection by direct contact was attempted by isolating infected patients to the north wing and uninfected patients to the south wing of the unit. All staff were similarly segregated so that contact between the two groups would not take place during any term of duty. During the subsequent 12-month period, and for the first time, patients with extensive burns passed through their treatment in the unit without any evidence of *Pseudomonas* infection; periods of treatment were from 80 to 120 days. Further confirmation of reduction of cross-infection was found when pyocine types from the year before were compared with those in the year after isolation of the two groups (Table 5). The chain-like pattern of infections during 1966 was completely broken after elimination of direct contact and was replaced by a random scatter of pyocine types. This freedom from cross-infection continued for the next 12 months and the *Pseudomonas* infections were reduced from 41 % in 1966 to 28 % (20 out of 72 admissions) in 1967. During the early part of 1968 the unit became short of staff and strict isolation of the two groups was not possible. Once again a chain pattern of infections involving several patients with a single pyocine type of *Pseudomonas* emerged, which was a clear indication of cross-infection. Only when more staff became available was the chain pattern of infections broken and replaced by a random scatter of pyocine types.

A further reduction of *Pseudomonas* infections was experienced during 1968. Out of a total of 73 patients treated in the unit only 15 (20 %) became infected, and this, when considered with other evidence, confirmed that some control of cross-infection by direct contact had been achieved.

DISCUSSION

When patients with *Pseudomonas*-infected wounds are nursed in hospital this organism becomes established and can be recovered from many parts of the environment. Given an environment free from *Pseudomonas* at the outset, the admission of burns patients with infected wounds provides known sources of this organism and permits its environmental distribution to be studied. It seems likely therefore that the question of whether pseudomonas must become a resident or not could be answered. As a result of controlled disinfection, daily bacteriology failed to show resident *Pseudomonas* in any part of the Burns Centre, and after 3 years we concluded that the persistence of this organism was not inevitable. If it should become established it could be got rid of by daily disinfection. These studies have shown that *Pseudomonas* was capable of being airborne for prolonged periods and was being distributed in the air over wide areas from confirmed sources. The spread of *Pseudomonas* from patients' cubicles was controlled by using topical creams to prevent it becoming airborne, but during treatment of patients in the dressings suite and the operating theatre the most effective method of control was achieved by disinfectant aerosol mists followed by a surface-disinfectant wash. Although we undoubtedly experienced cross-infection during the first year, this was considerably reduced by isolation and segregation procedures, and our results seemed to show that persistence of *Pseudomonas* in a Burns Centre is not inevitable, that the main cause of cross-infection is by contact, and that control of cross-infection is possible.

During the four years 1966–9 inclusive the annual numbers of confirmed deaths from *Pseudomonas* septicaemia were 4, 1, 1 and 0 respectively.

I wish to record my profound appreciation to Professor J. W. McLeod for his constant interest and encouragement over the years. My sincere thanks are due to all those who have contributed so much to these studies: Mr T. L. Barclay, Dr J. A. D. Settle, the Sisters and staff of the Regional Burns Centre without whose cooperation this work would not have been possible, and Dr M. T. Parker for his help and advice on pyocine typing. I also have to acknowledge with grateful thanks the valuable assistance I have received from Mr F. Swift, Mrs M. Jordan and Mrs M. Smailes.

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Studies of respiratory viruses in personnel at an Antarctic base

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SUMMARY

Thirteen men wintering on an Antarctic base were isolated from other human contact for 10 months. During this period Coxsackievirus A21 and later influenza A2 virus were administered to some of the men. Serum samples were collected from each of the men at monthly intervals.

Coxsackievirus A21 produced symptoms and apparently spread to uninoculated men. It also appears that repeated re-infections occurred and that the virus persisted in this small community for most of the period of isolation. HI antibody responses in the absence of neutralizing antibody responses seem to be transient.

The vaccine strain of influenza virus induced antibody responses but did not cause symptoms. There was no evidence of spread to uninoculated men.

Antibody titres against influenza C, parainfluenzaviruses 1 and 2 and coronavirus OC43 did not fall significantly during isolation.

An outbreak of respiratory illness occurred at the end of isolation and its origin was traced. No causative agent was detected.

INTRODUCTION

Stonington Island is a British Antarctic Survey base situated on the south-west coast of the Antarctic Peninsula. The men who spend the winter there are isolated from contact with other personnel for periods of 10 or more months a year. Such an isolated population provides a good group in which to study the spread of respiratory viruses in the absence of extraneous infections by other agents, and to study the persistence of antibody against respiratory viruses in the absence of any likelihood of exposure to them. It is also of interest to examine the possible effects of a cold environment on the symptoms produced by respiratory viruses.

This paper describes two trials at Stonington in which men were inoculated with Coxsackievirus A21 and influenza A2 virus, strain Leningrad/4/65. Clinical

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symptoms of the men and their antibody status against these and other respiratory viruses were studied throughout their stay at the base, from February 1968 to March 1969.

MATERIALS AND METHODS

Population studied

The wintering party at Stonington in 1968 consisted of thirteen men. Eight men left England in September and October 1967, and arrived in February 1968. They were joined, on the way, by one man from a sub-antarctic base. The other four men had already spent 1 year at Stonington. The thirteen men were isolated from other humans until an aeroplane landed on 9 December 1968. Between 13 March and 8 July 1968, six men were trapped on a small ice shelf to the north (the Jones Ice Shelf) and were therefore not available for study. The base hut was compact, particularly the bunkroom, which was divided into three cubicles, 8 ft. × 7 ft. × 7 ft. high, each accommodating four men. Over 100 husky dogs were also kept on the base.

Viruses

Coxsackievirus A21 in the form of tissue culture fluid from human embryo kidney cells infected with nasal washings from volunteers at the Common Cold Unit, and influenza A2/Leningrad/4/65 in the form of lyophilized allantoic fluid, were transported and stored at temperatures between -7 and $-15°$ C. The influenza virus was a vaccine strain originally obtained from Professor A. A. Smorodintsev, Leningrad. It had one passage in a human volunteer and two passages in eggs at Salisbury (Beare, Bynoe & Tyrrell, 1968).

Administration

On 12 April 1968, after two months in isolation, Coxsackievirus A21 was given to four of the seven men then at the base. The others were given a placebo consisting of phosphate buffered saline (PBS) containing flour and red ink. Administration of virus and placebo was double blind. Virus was diluted 1/10 in PBS and 2 ml. inoculated intranasally. The original virus pool had a titre of 10^4 TCD50 per ml. in human embryo kidney cells before leaving England, on 12 September 1967.

Influenza A2 virus was given to seven of the 13 men on base on 26 July 1968. The remainder were given placebo (PBS containing powdered milk). Each ampoule of virus was reconstituted in 10 ml. PBS and 2 ml. inoculated intranasally. Administration of virus was again double blind. When no symptoms developed, the code was broken and the six men who received placebo on 26 July were given virus on 15 September. Each ampoule of virus contained 10^6 EID50 when titrated in embryonated eggs before leaving England.

Samples of each virus were brought back to the U.K. in July 1969 and shown to be viable. Coxsackievirus A21 was recovered in Hela cells and A2/Leningrad/4/65 in embryonated eggs.

Collection of sera

Sera were collected from six men before leaving England and from the other seven soon after arrival at Stonington. Thereafter sera were collected at intervals of 4–6 weeks, throughout the following 12 months. Sera were stored at 4° C. in a temperature-controlled box at the base, and at − 9 to − 15° C. on the return voyage.

Serological tests

Sera were tested for haemagglutination-inhibiting (HI) antibody against Coxsackievirus A21 by the microtitre method, using human foetal group O cells (Chapple, 1966) and for neutralizing antibody by a microtitre colour test (Stott & Tyrrell, 1968). Also using the microtitre method, HI antibodies were estimated against influenza A2/Leningrad/4/65, influenza A2/HK/1/68, influenza C, parainfluenza viruses 1 and 2 and coronavirus OC 43. Complement fixing (CF) antibodies were titrated against influenza viruses A, B and C, parainfluenza virus 1, respiratory syncytial virus (RSV) and the adenovirus group. Sera for HI tests were treated with cholera filtrate, absorbed with chicken red cells and inactivated at 56° C. for 30 min, except in the case of the OC 43 tests, where they were diluted with PBS and inactivated at 65° C. for 30 min. Sera for neutralization and CF tests were inactivated at 56° C. for 30 min.

Serum globulin estimations

Serum globulin values (IgG, IgA, IgM) were estimated in the sera collected throughout the year from four subjects, using a gel-diffusion technique (Immunoplate, Baxter Laboratories).

RESULTS

Clinical trials during isolation

Coxsackievirus A21 trial

On 12 April 1968, four of the seven men on the base were given Coxsackievirus A21; the remainder received a placebo (Table 1). All four men given virus and one of the men given placebo developed symptoms. The onset of illness in the man given placebo (36 hr. after the others) suggests transmission of infection from one of the men given virus. The two men who did not develop symptoms (subjects 6 and 7) were the only men in this group who had detectable neutralizing antibodies and who had HI titres higher than 12 before inoculation. Four-fold or greater rises in HI antibody titres were detected in all five men who became ill, but a significant rise in neutralizing antibody titre was found only in subject 10. The incidence of symptoms in the men who were infected is shown in Table 2.

Influenza A2/Leningrad/4/65 trials

Seven of the 13 men on base were given influenza A2 virus on 26 July (Table 3). The other six received placebo. None of the men developed any symptoms, but three of those given virus (subjects 1, 9 and 13) developed four-fold or greater rises

Table 1. *Clinical and serological results of Coxsackievirus A 21 trials*

(Virus administered on 12 April 1968.)

		Clinical response				Antibody titres*					
						HI			Neutralizing		
Subject	Inoculum	Symptom	Severity	DUR	DO	Apr.	May	July	Apr.	May	July
4	Virus	URTI	Mild	6	1·5	< 6	12	72	< 4	< 4	< 4
5	Placebo	URTI	Mild	3	3	6	24	6	< 4	< 4	< 4
6	Placebo	None	—	—	—	48	24	48	256	250	256
7	Placebo	None	—	—	—	18	18	36	16	32	64
8	Virus	URTI	Mild	5	1·5	< 6	48	6	< 4	4	4
10	Virus	URTI	V. mild	4	2·5	12	48	9	4	16	32
13	Virus	Diarrhoea	V. mild	3	2·5	6	24	18	< 4	< 4	< 4

DUR = duration in days,
DO = day of onset after inoculation,
URTI = upper respiratory tract illness.

* Expressed as reciprocal of highest dilution of serum showing 50 % inhibition of haemagglutination of 10–100 50 % tissue culture infecting doses (TCID 50) of virus. Sera were collected on 12 April, 15 May and 26 July 1968.

in HI antibody titre during the following 7 weeks, indicating that they had been infected. Two men (subjects 2 and 6) had antibody rises of < 6 to 6 eventually rising to 12. The remaining two men who were given virus (subjects 7 and 11) had pre-inoculation titres of 48 and 144 respectively. None of the men given placebo developed significant rises in antibody titre.

Table 2. *Incidence of symptoms on Coxsackievirus A21 trial (5 men infected)*

Symptom	No. of men with symptom
Coryza	4
Blocked nose	4
Headache	4
Sore throat	1
Pyrexia	2
Malaise	4
Myalgia	2
Abdominal colic	2
Diarrhoea	4

Table 3. *Serological results of influenza A2 Leningrad trials*

	Inoculum		HI antibody titres in indicated serum*				
Subject	26 July	15 Sept.	1	2	3	4	5
1	Virus	Placebo	< 6	24	24	—	24
2	Virus	Placebo	< 6	6	6	—	12
3	Placebo	Virus	< 6	< 6	24	—	24
4	Placebo	Virus	< 6	< 6	24	—	12
5	Placebo	Virus	12	12	48	—	48
6	Virus	Placebo	< 6	6	—	12	12
7	Virus	Placebo	48	36	36	—	48
8	Placebo	Virus	6	6	—	6	6
9	Virus	Placebo	< 6	24	24	—	18
10	Placebo	Virus	< 6	< 6	—	9	9
11	Virus	Placebo	144	96	96	—	96
12	Placebo	Virus	12	24	24	—	36
13	Virus	Placebo	< 6	18	—	24	18

*1 serum collected 26 July,
2 serum collected 15 September,
3 serum collected 7 October,
4 serum collected 27 October,
5 serum collected 2 December.

Titres expressed as reciprocal of dilution of serum showing 50% inhibition of haemagglutination.

On 15 September, the six men given placebo on 26 July were given virus, and the other seven were given placebo. Again, none of the men developed any symptoms. Three of these (subjects 3, 4 and 5) developed significant antibody responses. One (subject 10) had an antibody rise from < 6 to 9 eventually reaching 12. The

remaining two subjects (8 and 12) showed no rises. Subject 12 had a pre-inoculation titre of 24 and subject 8, who had a pre-inoculation titre of 6, misunderstood instructions and swallowed his inoculum before it could be given intranasally.

Survey of antibody titres and globulin values during isolation

Influenza A2/Hong Kong/68

Only one man (subject 11) had detectable HI antibody at the beginning of the period of isolation. His initial titre of 8 was maintained throughout the year. One other man (subject 7) apparently had a rise in HI antibody titre from < 8 to 16 between July and September 1968. No other significant changes were detected.

Influenza C

All 13 men had HI antibody (titres 8 to 128) against influenza C at the beginning of isolation. Titres stayed constant for the ten months in all the men except subject 12, whose titre fell from 64 to 16.

Parainfluenza 1

Five of the six men who were bled in England before they left showed four-fold or greater rises in HI antibody titre by the time they arrived at Stonington. This suggests that there was an outbreak of parainfluenza 1 on the ship, though no clinical symptoms were reported. All 13 men had HI antibodies against parainfluenza 1 (titres 8 to 256) at the beginning of isolation, and only subject 4 showed a significant fall in titre (256 to 64) during the following 10 months.

Parainfluenza 2

Three men had no detectable HI antibody in March 1968. Three of the 10 men (subjects 7, 8 and 11) who had antibody showed a four-fold reduction in titre by December. In subject 7, the fall in titre occurred in April; in the other two cases, titres fell in the last three months of isolation.

Coronavirus OC 43

Coronavirus HI antibody was present in 10 out of 11 subjects examined (titres 10 to 80). All of these 10 subjects maintained their titres without significant change.

Coxsackievirus A 21

Two men (subjects 2 and 12) had eight-fold rises in HI antibody titre between leaving England and arriving in Antarctica. Apart from these two cases and the five antibody responses directly associated with the trial, there were ten significant HI rises: a six-fold rise in subject 8 between July and September; four- to eight-fold rises in subjects 3, 5, 11 and 12 between September and October; four-fold rises in subjects 1, 10 and 13 between December 1968 and February 1969; six-fold rises in subjects 7 and 8 between February and March 1969 (Table 4).

A four-fold or greater fall in HI antibody titre was detected on 12 occasions. Ten

of these falls occurred in eight of the nine subjects whose neutralizing antibody titres were four or less. Two falls were detected in the four men who had neutralizing antibody titres of eight or more, and in both cases these were accompanied by falls in neutralizing antibody.

Table 4. *Haemagglutination-inhibition titre rises against Coxsackievirus A* 21 *in the personnel at Stonington Island, September* 1967 *to March* 1969

Subject number	Sept. 67 to Feb. 68	Apr. 68 to Jul. 68	Jul. 68 to Sept. 68	Sept. 68 to Oct. 68	Dec. 68 to Feb. 69	Feb. 69 to Mar. 69
1	—	—	—	—	4	—
2	8	—	—	—	—	—
3	—	—	—	4	—	—
4	—	> 12*	—	—	—	—
5	—	4	—	4	—	—
6	—†	—	—	—	—	—
7	—†	—	—	—	—	6
8	—†	> 8*	6	—	—	6
9	—†	—	—	—	—	—
10	—	4*	—	—	4	—
11	—	—	—	8	—	—
12	8	—	—	6	—	—
13	—†	4*	—	—	4	—

* Subjects given virus during the Coxsackie A 21 clinical trial on 12 April 1968.
† No preliminary serum taken in September 1967.
The figures are not titres, but show maximum rise of titre; i.e. 8 = eight-fold rise.

Influenza A 2/Leningrad/4/65

Apart from changes in HI antibody titres which could be associated with the trials described above, there were no significant changes in titre during the seven months after the trials.

Serum globulins

Serum globulin estimations were made on serial sera from four men to see if fluctuations in globulin were related to any of the antibody titres measured. Two sets of sera were taken from men spending a second year in Antarctica and two from men spending their first year. One of each pair was with a party which was partially starved for 6 weeks. Although some low values and fluctuations were recorded, they bore no obvious relation to the antibody titres of any of the four subjects.

Clinical symptoms during isolation

Apart from the symptoms associated with the Coxsackievirus A21 trial, respiratory symptoms occurred in only one man during the period of isolation: subject 11 had intractable purulent catarrh and, on two occasions in the spring, developed a left maxilliary sinusitis which was relieved only by puncture and irrigation of the sinus.

Upper respiratory disease after isolation period

Epidemiology

Details of visitors to Stonington after the 10-month period of isolation (Table 5) show that no respiratory illness developed in men on the base until the arrival of the R.R.S. *John Biscoe* on 13 February. The next day an epidemic began among

Table 5. *Details of visitors to Stonington base at the end of 10-month isolation period*

Date	Transport	No. persons	Origin	Duration of visit	Result in base personnel
Dec. 1968					
9	Aircraft	2*	Adelaide Is.	0·5 hours	Isolation broken, no symptoms
16	Helicopter	6	HMS *Endurance*	6 days	No symptoms
Jan. 1969					
28	Aircraft	2	Adelaide Is.	1 hour	No symptoms
Feb. 1969					
13	Ship	3†	See Table 6	1 year	On 14th and 15th upper respiratory infection in 7 of 10 men originally on base

* Aircraft crew had themselves been isolated for about 7 weeks.
† All three men convalescing from respiratory illnesses.

Table 6. *Spread of upper respiratory tract infection carried on R.R.S.* John Biscoe

Date	
Jan. 1969	
31	Argentinian ship, *Bahia Aguirre*, visits Argentine Is. (300 miles north of Stonington). First contact of island with outside world for 4 months.
Feb. 1969	
1	Ship (R.R.S. *John Biscoe*) collects man from Argentine Is.
2	Man on ship develops upper respiratory symptoms.
3	Ship arrives Adelaide Is. (70 miles north). Upper respiratory symptoms develop in most of base personnel on board and in half the officers, but none of the crew.
5	All base personnel at Adelaide Is. develop respiratory symptoms. Aircraft from Adelaide Is. visits Fossil Bluff (300 miles south).
7	Respiratory symptoms develop in men at Fossil Bluff.
13	Ship arrives at Stonington. Three men disembark, all recovering from respiratory illness. Ship leaves with three base personnel from Stonington.
14	Respiratory symptoms develop in six of ten base personnel remaining at Stonington.
15	Ship returns to Stonington. Two of three men collected on 13th disembark. Both have respiratory symptoms. Ship leaves. Symptoms also develop in seventh man on base.
17	HMS *Endurance* stays 4 hr. at Stonington. Two days later respiratory symptoms develop on board.
19	Ship returns to Stonington. Third man collected disembarks with severe upper respiratory symptoms.
25	Clinical observer leaves for Adelaide Is. All ill men are now convalescent.

the 16 men who were on the base. Three of them had landed the previous day from the ship. All three were recovering from respiratory infections. Ten of the 13 men who had wintered at Stonington became ill. The three who did not were all completing the second year of their tour. The symptoms experienced by three of the 13 affected were clinically consistent with 'influenza', seven with a 'severe' cold and the remaining three with a 'moderate' cold (Tyrrell, 1965). The movements of the ships and aircraft involved in the spread of respiratory illness which eventually arrived at Stonington can be followed in Table 6 and the map of the Antarctic peninsula (Fig. 1).

Complement-fixing antibody levels

A limited number of the sera taken in February 1969 were tested for the presence of CF antibody against influenza viruses A, B and parainfluenza 1, respiratory syncytial (RSV) and an adenovirus. This was done in the hope of identifying the

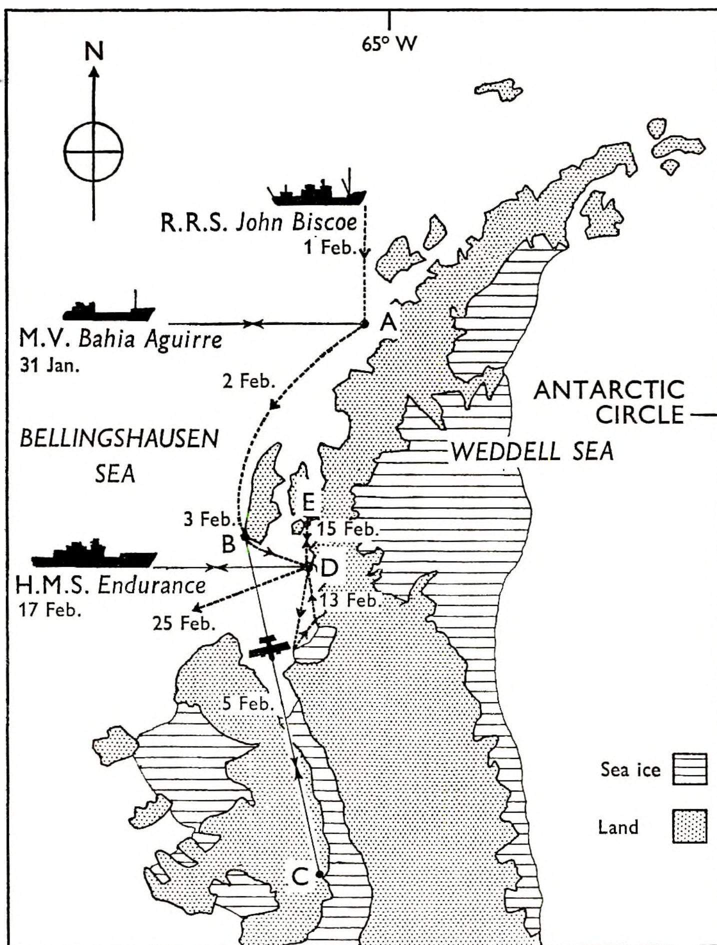

Fig. 1. The Antarctic Peninsula: Movements of ships and aircraft between 31 January 1968 and 25 February 1969. Key. A. Argentine Is.; B. Adelaide I.; C. Fossil Bluff; D. Stonington I.; E. Jones Ice Shelf.

agent responsible for the epidemic of respiratory disease which occurred after the isolation period and before the relief ship left. The sera were uniformly negative, but they were taken only a week after the start of the epidemic.

DISCUSSION

The Coxsackievirus A21 trial showed that men at Stonington Island were infected with similar frequency, and had similar symptoms, to volunteers infected in England and the United States (Parsons, Bynoe, Pereira & Tyrrell, 1960; Spickard, Evans, Knight & Johnson, 1963; Buckland, Bynoe & Tyrrell, 1965). The unusually high incidence of gastro-intestinal symptoms parallels observations made during an outbreak of respiratory disease at an Australian Antarctic base (Cameron & Moore, 1968). Here they were attributed to the diet, but a high incidence of gastro-intestinal symptoms has also been recorded during outbreaks of respiratory disease by other polar workers (Abs, 1930; Paul & Freese, 1933).

The infection of subject 5, the only man on base at that time who had no detectable neutralizing antibody and who received placebo, suggests that the virus could spread readily under the conditions at Stonington. Buckland *et al.* (1965) noted that this virus only spread successfully under conditions of the barrack room or in crowded communities (Fukumi, Nishikawa, Sonoguchi & Shimizu, 1962; Chapple, 1966). Living conditions at Stonington were more confined than those of the barrack room. The high neutralizing antibody titres of subjects 6 and 7 appear to be protective. This is probably the reason why they were the only men on base at the time of the trial who did not become infected. It is noteworthy that subject 6, who had the highest titre of neutralizing antibody, had recently spent two years in New Guinea, and in a world-wide survey Micronesia was the area found to have the highest percentage of people with antibody to Coxsackievirus A21 (Chapple, 1966). Illness due to Coxsackievirus A21 is usually confined to those without neutralizing antibody, but such antibody does not prevent further symptomless infection (McDonald, Miller, Zuckerman & Pereira, 1962; Johnson, Bloom, Mufson & Chanock, 1962; Spickard *et al.* 1963; Oei & van der Veen, 1967).

Our findings indicate that a rise in HI antibody, when unaccompanied by a rise in neutralizing antibody, may be transient, and does not appear to protect against further symptomless infection; evidence of such transience was recorded without comment by Buckland *et al.* (1965). Furthermore a single infection, in subjects without pre-existing antibody, appeared to be insufficient to stimulate the production of much neutralizing antibody; the same conclusion was drawn by Parsons *et al.* (1960). Other studies, however, have shown that significant rises in neutralizing antibody may occur after infection in 60–70 % of people with no pre-existing neutralizing antibody (Spickard *et al.* 1963; Oei & van der Veen, 1967).

Only one HI antibody rise occurred against influenza A2/HK/1/68 and none against influenza C, parainfluenzaviruses 1 and 2 or coronavirus OC 43 during the period of isolation. Thus the HI antibody rises against Coxsackievirus A21 which were detected after July (three months after the trial) appear to be specific and most probably due to infections with this virus. The fact that variations in

globulin values did not correlate with antibody titres is further evidence for the specificity of the rises in antibody titres. The five antibody rises which occurred after December 1968 could have been due to infection from outside contact. However, the five antibody rises which occurred during isolation suggest that infections and reinfections continued to occur, or that prolonged faecal carriage stimulated delayed antibody response. Volunteer studies in men with no pre-infection antibody have shown that virus is excreted from the throat for up to 40 days after infection (Johnson *et al.* 1962). Such a long period of excretion would allow time for reinfection by the respiratory route. Although Coxsackievirus A21 is most frequently isolated from the throat, it is also found in faeces (Lennette, Fox, Schmidt & Culver, 1958; Parsons *et al.* 1960; Spickard *et al.* 1963). Hence virus could spread by the faecal-oral route. It is also possible that the coprophagic habits of the husky dogs could have caused them to become infected and involved as vectors in the epidemiology of Coxsackievirus A21 at Stonington. This latter possibility is currently under investigation. It is unlikely that prolonged faecal carriage stimulated a delayed antibody response, since in several men antibody titres fell before showing a rise and virus has only been found in the faeces of infected men for a few days (Spickard *et al.* 1963).

In the trials with influenza A2/Leningrad/4/65, men were infected when their pre-infection HI antibody titre was 12 or less, yet there was no evidence of any illness or spread of the virus. In similar trials at Salisbury with the same pool of virus, 13 of 18 volunteers with HI antibody titres of 24 or less were infected (Beare *et al.* 1968). However, nine of the infected volunteers at Salisbury developed symptoms. The influenza virus failed to spread because it was vaccine strain attenuated by passage in eggs (McDonald, Zuckerman, Beare & Tyrrell, 1962).

Although it is difficult to compare clinical results obtained by different observers at Salisbury and Stonington, the available evidence suggests that fewer symptoms were produced by the infection in the Antarctic. Evidence from animal experiments suggests that sudden exposure to cold results in more severe virus infection (Pasteur, Joubert & Chamberland, 1878; Walker & Boring, 1958). However, there are reports that Coxsackievirus infections were less severe in mice adapted to cold over several weeks than in controls (Marcus, Miya, Phelps & Spencer, 1963; Marcus & Miya, 1964).

Antibody against the other respiratory viruses appeared to persist with little fluctuation in most cases. The suggestion that men returning from the Antarctic experience severe respiratory disease because their antibody titres have fallen is not supported by our results. Antibody titres against rhinoviruses were not measured because of the multiplicity of serotypes and the limited quantity of serum. The occurrence of subclinical infections with parainfluenzavirus 1 while the ship was travelling south supports the results of Parrott *et al.* (1962). They showed that, while pre-existing antibody mitigates symptoms, it does not preclude reinfection. More recent evidence suggests that the presence of nasal antibody protects against the appearance of symptoms (Smith, Purcell, Bellanti & Chanock, 1966).

The occurrence of a respiratory epidemic after the end of a period of isolation is a common observation by those studying remote communities (Paul & Freese, 1933; Cameron & Moore, 1968). In this case a causative agent could not be identified. Unfortunately the latest that sera could be collected was only 7 days after symptoms appeared in the men, and this is rather early to expect definite changes in antibody titres. However, some indication of infection might have appeared and, since there was no evidence that the common myxovirus agents were implicated, it is possible that the disease was caused by a rhinovirus. Proof of this would require virus isolation results, and so far it has proved impossible to store and transport the necessary specimens in a suitable condition for study.

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Comparative trial of three heterologous anti-tetanus sera

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SUMMARY

The three heterologous anti-sera currently provided for tetanus prophylaxis have been compared with reference to the production of untoward reactions in 498 patients, and to the blood antitoxin concentrations produced in 76 patients. Equine serum, although giving rise to more reactions, was the only effective agent in terms of the levels and duration of serum antitoxin concentration produced. The local response to a test dose of any of the three sera is not a reliable guide to immediate or late general reactions.

INTRODUCTION

There is universal agreement that the essential factor in the prevention of tetanus following injury is prompt and adequate surgical attention. By contrast, conflicting views have been expressed in recent years regarding the place of equine anti-tetanus serum as an additional, specific prophylactic agent. For low-incidence areas, such as Britain and the United States of America, Rubbo (1966) based a strong case against the continued use of equine antiserum on the known dangers of morbidity and mortality that may follow its administration. In addition, hypersensitivity to equine serum results in accelerated elimination and reduced efficacy of the serum in some patients. Alternatives to the use of equine antiserum for the prophlaxis of tetanus include the administration of prophylactic antibiotics and the use of antisera developed in other animals or in man.

Experimental work with animals has cast doubts on the degree of protection afforded by penicillin to patients with wounds that have received delayed or inadequate attention (Smith, 1964). Important considerations here include the possibility of coexistent pyogenic infection with organisms that produce penicillinase, the persistence of resistant tetanus spores, and inadequate access of antibiotics to areas of ischaemic tissue. Misgivings have understandably been expressed concerning the prophylactic administration of antibiotics for this purpose, as in other situations such use is considered inadvisable. Moreover, there is no general agreement on either the choice of antibiotic or what constitutes a fully protective dosage schedule. Most authorities accept that the alternative or additional protection of antiserum should be given in some cases. As a result of these different considerations various codes of practice now exist for the prophylaxis of tetanus and a bewildering series of recommendations confronts the casualty officer. Despite

guidance given by the British Ministry of Health Advisory Group (1964) on this subject, we feel that the situation requires further clarification particularly with regard to the use of prophylactic antiserum. At present three heterologous anti-tetanus sera ('ATS') of equine, bovine and ovine origin, are produced in this country whereas a supply of homologous anti-tetanus globulin (ATG of human origin), which would resolve many of the problems, is not yet generally available in Britain. It is not our wish to enter here into the controversy regarding the relative place of homologous and heterologous sera. The purpose of the present paper is to present our experience of the three heterologous sera (equine, bovine and ovine ATS) that are currently available and officially recommended for clinical use. Our observations concern untoward reactions produced, and the serum antitoxin titres achieved and maintained in the patient.

MATERIAL AND METHODS

Pepsin refined bovine, equine and ovine anti-tetanus serum was supplied in ampoules by the Wellcome Research Laboratories. Each ampoule contained a single dose of 1500 i.u. antitoxin labelled A, B or C respectively, but the identifying code was unknown to any member of the clinical team. Ampoules containing the vehicle without any immune globulin were also available as controls. Patients were considered for the trial consecutively as they presented themselves at the Accident and Emergency Department during the period of January to June 1968. All those with a definite history of previous active immunization were treated with a booster dose of tetanus toxoid, and all those under 21 years of age were excluded from the trial. All other patients for whom specific tetanus prophylaxis was necessary were considered eligible. The form and purpose of the trial were explained to each individual and only those who consented were included. A careful history was taken from each subject regarding previous immunizations and allergies and this information was later checked as far as possible with the Public Health Authorities and with the general practitioner concerned. The basis of each patient's treatment was surgical toilet of the wound and administration of ATS, but a definite history of allergy was regarded as a contra-indication to serum prophylaxis. Every patient received an intramuscular injection of adsorbed tetanus toxoid. Antibiotics were not prescribed as prophylactic agents against tetanus, but were used if considered clinically desirable when pyogenic infection was diagnosed.

The antiserum was selected by a predetermined random pattern on a weekly rotation. Each patient was given a subcutaneous test dose consisting of 0·1 ml. of the 'serum of the week' (A, B or C). Simultaneously a control injection of the vehicle was given at a corresponding position on the opposite forearm. The patient was observed over a period of 30 min. for the development of any local or general reaction, and the findings were recorded. A positive local reaction was arbitrarily defined as a wheal measuring 1 cm. or more in diameter. Any local reaction less than this was recorded but the full dose of the serum was administered by intra-muscular injection. If the local response was a wheal greater than 1 cm. in diameter, test doses of the other two sera were given, and the patient received the

full dose of the serum to which the local reaction was least marked. As the trial progressed it was confirmed that a local reaction consisting of a wheal greater than 1 cm. was not evidently associated with any increased risk of an immediate general reaction and in many cases in the later part of the trial the full dose of the serum was given despite such local effect. The position of each injection was standardized and marked with indelible ink, and the patient was reviewed on his return for wound treatment. Any reaction or history of reactions was recorded and return visits arranged for the patients concerned. The general practitioners in the region, who had previously been consulted through the local medical committee, were asked to report any possible reactions to ATS that developed in their patients included in the trial. A suitable form was supplied for the return of this information. The patients themselves were contacted by postal survey at the end of the trial to obtain information about any late reactions which had not been observed by ourselves or reported by the family doctor.

Samples of venous blood were taken from approximately every sixth patient in the trial for assay of antitoxic activity before the first injection and subsequently on the 5th, 10th, 14th and 21st days thereafter, or as close to these days as could be arranged. Defaulters were visited at their homes to complete the taking of blood samples. The serum was separated immediately and dispatched to the Wellcome Research Laboratories where the antitoxin content was assayed by *in vivo* titration using the mouse subcutaneous method (Glenny & Stevens, 1938). At the end of the trial, the potency of the three antisera was rechecked, and no significant deterioration had occurred.

RESULTS

The trial included 498 patients on whom clinical data were completed, and antitoxin titres were followed in 76 of these patients. The data were analysed by the Wellcome Foundation on an ICL computer; 205 patients received serum A (bovine), 148 serum B (equine), and 145 serum C (ovine).

REACTIONS ATTRIBUTABLE TO SERUM

The reactions produced by the test doses were compared with those produced by the injection of vehicle alone. It was found that a zone of erythema up to 1 cm. in diameter was occasionally produced by the vehicle and it was, therefore, concluded that such a local reaction was not necessarily attributable to the protein in the injection and could be ignored. Local reaction to the test dose was recorded by measuring the diameter of the wheal on three occasions during the 30 min. following injection. Because of the work load in the Accident Department precise times of recording could not be achieved and three time brackets of 5 min. were therefore used (Table 1). Reactions greater than 1 cm. were more common with equine serum than with either bovine or ovine (7·4, 4 and 2·8 % respectively) but this difference is not statistically significant.

There was no immediate general reaction to a test dose of any serum or to a full dose of bovine or ovine serum; a full dose of equine serum may have been

responsible for an immediate, general reaction in one patient who felt faint and nauseated, became hypotensive and was seen to hyperventilate. This reaction was brief and self limited. The patient had not shown any reaction to the test dose.

Table 1. *Incidence of local wheals after subcutaneous injection of* 0·1 *ml. of antiserum*

Serum	No. of cases	No. showing induration of ⩾ 1 cm. in diameter at 15–20 min.	20–25 min.	25–30 min.
Bovine	205	4	6	8 (4)
Equine	148	13	12	11 (7·4)
Ovine	145	2	4	4 (2·8)

The figures in parentheses are the percentage positive after 25–30 min.

Table 2. *Incidence of late reactions (local and general) occurring after a full dose of the three antisera*

Serum	No. of patients	No. of patients showing reactions: Total	Local	General	Both
Bovine	205	25 (12)	18	7 (3)	0
Equine	148	42 (28)	26	16 (11)	2
Ovine	145	17 (12)	8	9 (6)	2
All sera	498	84 (17)	52	32 (6)	4

The figures in parentheses are percentages.

Table 3. *Details of late general reactions occurring after a full dose of the three anti-sera*

Serum	Adenitis	Rash	Arthritis	Other	Days off work
Bovine (7 cases)	3	4	1	—	2
Equine (16 cases)	4	9	3	4 (giddiness and headaches)	11 (1 case off 7 days with skin rash)
Ovine (9 cases)	2	5	1	2 (sickness and headaches)	½

Of the delayed reactions, local induration in the buttock was by far the commonest, and such local reactions to the full dose were significantly more common after equine serum (Table 2).

Delayed general reactions, or symptoms that could be attributed to this cause were observed in 7 out of 205 patients given bovine serum, 16 patients out of 148 given equine serum and in 9 patients out of 145 given ovine serum (Table 2). The increased incidence after equine serum is again significant. Of these 32 patients

only 2 had shown a local reaction to the test dose. More detailed analysis of the late general reactions is summarized in Table 3. Equine serum was responsible for the loss of 11 working days whereas bovine and ovine serum caused the loss of 2 days and one half day respectively. (The morbidity for equine serum was considerably increased by one patient who was away from work for 7 days on account of a skin reaction.)

Efficacy

A serum antitoxin concentration of more than 0·01 units per ml. was regarded as the minimum protective concentration (King, Kaiser, Lempke & Ruster, 1963; McComb, 1964; White *et al.* 1969). The necessary duration of such a concentration will depend on the wound concerned but a period of 10–14 days would give protection in the majority of cases.

Table 4. *Percentage of patients with predicted serum anti-toxin titres greater than* 0·01 *units per ml. at various times after the injection of* 1500 *units of anti-toxin*

Serum	Day 1	Day 2	Day 7	Day 10	Day 14
Bovine (22 patients)	68	64	55	22·7	14
Equine (16 patients)	100	100	100	100	88
Ovine (13 patients)	62	62	23	8	15

Analysis of the data obtained was complicated by two factors. First, our decision to give all patients in the trial simultaneous active immunization. When the passive protective potency of the serum came to be assessed, of the 76 patients in whom adequate serial assays were available 25 were excluded because of either high initial serum antitoxin titres or an early secondary immune response to the toxoid injection. Secondly, there was inevitably some variation in the days after injection on which blood samples were taken so that direct comparison of the titres for the three sera would be invalid. Bias due to this factor was removed by utilizing regression equations, calculated by plotting the logarithms of the titres against the elapsed time, to predict responses at a fixed time and then analysing these predicted responses. Table 4 shows the percentage of subjects who would be predicted to have protective antitoxin titres at various times as calculated from the data for the 51 'valid' cases. There is a very obvious and statistically significant difference ($p = 0{\cdot}01$) between the patients given equine serum and those given the other two sera.

DISCUSSION

However strongly we may advocate universal active immunity as the proper approach to tetanus prophylaxis this ideal will not be readily achieved and the need for a passive protection of susceptible injured patients will continue to arise. A study of 3000 consecutive patients attending the Accident and Emergency Department in the early spring of 1968 showed that, of 629 patients considered

to require some form of prophylaxis against tetanus, 140 (22 %) claimed to have been actively immunized. In Leeds, after years of active campaigning, the proportion of patients attending the Accident Department in 1966 who had been actively immunized was 63·4 % (Ellis, 1967). In assessing the relative merits of serum and antibiotics in prophylaxis, the theoretical reasons that would justify preference for serum are set against dangers that attend the normal clinical use of equine anti-serum. These dangers will be markedly reduced if human serum is used but as this may not be widely available in Britain for some years it has been suggested that anti-sera should be prepared in animals other than the horse. Although Trinca & Reid (1967) found that bovine antitoxin produced few untoward reactions, the serum antitoxin concentrations achieved by the injection of bovine anti-serum in his patients were not recorded. In the present study we have confirmed that equine serum carried a higher morbidity than the other two sera though the loss of working days was much less than that found in Sheffield by Cox, Knowelden & Sharrard (1963), being 7 days per 100 patients in the present series as against 24 days per 100 patients in the Sheffield series.

More important, however, are the relatively low blood titres produced by the two sera which are at present officially provided by the Ministry of Health for use when equine serum has proved unsuitable. Whereas equine serum was predicted to give protective titres in 100 % of subjects at 10 days, bovine serum gave predicted protective titres in only 23 % at 10 days and ovine serum in only 8 %. It is difficult to explain this striking difference in efficacy. If any one of the three sera should have been eliminated more rapidly than the others this should have been the equine serum, because of previous exposure of many patients to this antigen. Moreover, accelerated elimination could not explain the findings because, in a small number of patients in whom serum antitoxin titres were assayed during the first 24 hr. there was no evidence of an adequate but earlier titre in the bovine and ovine groups, while the slope of the curves obtained by plotting log titres against elapsed time, and which could be taken to indicate the rate of decline of antitoxin titres, was similar for all three sera. Although the test groups were small it appears doubtful whether bovine and ovine anti-tetanus sera should continue to be regarded as acceptable alternatives to equine serum.

This conclusion is particularly important as there does not yet appear to be official support for increased production of human anti-tetanus globulin in this country. Our present evidence suggests that human ATG should now be at least the main alternative to equine serum and should entirely replace it as soon as is practicable. This view is supported by practice in other developed countries. An alternative solution might lie in means of reducing the antigenicity of horse globulin in man. Although some have claimed that ultracentrifuged preparations of bovine gamma-globulin have reduced antigenicity in mice (Dresser, 1962) and rats (Gery & Waksman, 1967) attempts by one of us to repeat this work with bovine and equine gamma-globulin in rats have so far been unsuccessful.

Although it has been standard teaching for many years that the value of a test dose of serum in predicting dangerous sensitivity lies only in the development of an immediate general reaction to the test dose, we have repeatedly observed

reluctance on the part of doctors to give a full dose of anti-serum to a patient who has shown a local wheal, or even erythema, in response to the test dose. Our experience confirms that the subcutaneous test dose is of no practical value in predicting delayed reactions to the sera (Laurent & Parish, 1962), and this finding applies equally to all three sera. This view has been contested by Binns (1961) who concluded, in a study of 3455 patients, that the local reaction was important in relation to the development of delayed general reactions. However, he did not state in detail the nature of these responses, save that two of them, occurring up to 50 min. after the test dose, were severe anaphylactic reactions. Certainly in our series, none of the delayed reactions was sufficiently severe to warrant the replacement of serum prophylaxis for that particular injury by any other form of therapy, and in only one of these cases was there an immediate reaction to the test dose of induration 1 cm. or more in diameter.

We should like to express our thanks to Dr A. H. Griffith of the Wellcome Research Laboratories, who provided the sera and helped in the planning of the trial, to Dr David Gall, who estimated the antitoxin titres of the blood samples, and to Mr D. A. Field who carried out the computer analysis of the results. We are grateful to Dr J. G. Collee for much helpful advice throughout the trial, to Miss Susan Murdoch, who was responsible for the documentation and secretarial work, and to the staff of the Accident and Emergency Department of the Royal Infirmary of Edinburgh for their helpful co-operation.

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Investigations of Allerton-type herpes virus infection in East African game animals and cattle

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SUMMARY

Neutralization tests with a strain (BA) of Allerton-type herpes virus, derived from a buffalo (*Syncerus caffer*) were carried out on 924 sera from 17 species of E. African game animals and on cattle sera from Tanzania (2001), Kenya (792) and Uganda (410).

Buffalo populations throughout E. Africa showed a very high rate of infection, with all animals over 2 years of age serologically positive. Antibody was present in some giraffe, waterbuck and hippopotamus sera and, less frequently, in impala, eland, bushbuck and oryx. Data are provided on the titres of positive samples; the mean titre of buffalo sera increased with age.

Cattle in many localities of N. Tanzania and S. Kenya showed a very high rate of infection, 85–95 % of sera from animals more than 2-years old containing antibody; the titres recorded were lower than those in buffaloes. Very high infection rates were also found in Karamoja and Teso (Uganda) and also in some other areas of Kenya, whilst a considerably lower incidence of infection was detected in W. Nile Province of Uganda and in central Tanzania. Differences in infection rates may have been related to herd size and husbandry practices.

It was shown that a wave of infection was probably spreading through cattle in N. Tanzania at about the same time as an outbreak of disease occurred in buffaloes and it is suggested that virus transmission may have been by biting flies.

No clinical signs attributable to the virus were reported in cattle but mouth lesions similar to those recorded in buffaloes, or nasal lesions, could have passed undetected. Allerton-type virus probably produces a range of clinical syndromes in cattle, closely resembling those associated with some herpes viruses in primates but infection is seldom related in the field to either pseudo-lumpy skin disease, mammillitis or stomatitis.

INTRODUCTION

In December 1969 an outbreak of disease, associated with an appreciable mortality, occurred in buffaloes (*Syncerus caffer* Sparrman) of the Serengeti National Park, Tanzania and in the contiguous Mara Game Reserve of Kenya. The disease affected predominantly young animals, 6–12 months of age, and was associated with the presence of well-defined areas of necrosis and ulceration in the

mucosa of the upper alimentary tract, including the tongue, oral cavity, oesophagus and rumen (Schiemann, Plowright & Jessett, to be published).

From a tongue ulcer in one yearling animal a virus (BA) was isolated in calf kidney and testis cultures which had the characteristic cytopathology of the Allerton-type of pseudo-lumpy skin disease virus (Alexander, Plowright & Haig, 1957). The quantity of virus recovered from diseased tongue tissue, $10^{4\cdot 2}$ TCD 50/g., left little doubt that it was the cause of the lesion and diagnostic cytopathological changes were seen in stained sections of the same ulcerations. Neutralizing antibody to the BA virus was present in 11 of 13 buffalo sera collected during and immediately after the outbreak.

The present paper describes extensive serological investigations, designed to establish the prevalence of infection with Allerton-type virus in East African game animal and domestic ruminant populations. A future communication will describe the identification of the buffalo strain as closely related to or identical with the prototype South African Allerton virus and record observations on its pathogenicity for experimental cattle.

MATERIALS AND METHODS

Virus strains

The origin of the buffalo Allerton-type virus (BA) is described above (Schiemann *et al.*, to be published).

Propagation of virus

Virus was grown in monolayers of primary bovine kidney (BK) cells produced by methods already described (Plowright, Herniman & Rampton, 1969). Before inoculation with virus, cultures were washed twice with phosphate buffered saline (PBS of Dulbecco & Vogt, 1954) to remove bovine serum incorporated in the growth medium; the maintenance medium contained 2 % unheated horse serum and was changed completely every 2 days. For stock virus preparation fluids were harvested when at least 80–90 % of the cell sheet exhibited cytopathic effects; small volumes of clarified culture fluids were stored at − 70° C. and thawed rapidly at 37° C. when required.

Virus assay

Virus preparations were diluted in 1·0 or 0·5 $\log_{10}$ steps, using culture maintenance medium as a diluent. Each of a suitable range of dilutions was inoculated into five BK tube cultures in a dose of 0·2 ml. and the tubes were afterwards rotated in roller drums at 36·5 ± 0·5° C. Microscopic examination for the typical syncytial foci produced by Allerton-type virus was carried out before medium changes and finally on the 5th and 6th days. Titres were calculated by the method of Spearmann–Kärber (Dougherty, 1964). The means and standard deviations for two virus stocks which were titrated 10 and 20 times were $10^{5\cdot 78 \pm 0\cdot 20}$ and $10^{5\cdot 67 \pm 0\cdot 15}$ respectively. The virus was stable over periods of at least 4 months at − 70° C.

Sera

Cattle sera from Tanzania and Kenya had been collected as part of a large-scale serological survey of rinderpest immunity, resulting from annual vaccination campaigns. Animals had been assigned to age groups on a basis of dentition, horn growth, body size and information provided by the owners, who were predominantly semi-nomadic pastoralists. Sera from small domestic ruminants were collected during a survey of rinderpest epizootiology in N. Tanzania.

Some game animal sera were obtained in the course of studies on the epizootiology of rinderpest, malignant catarrhal fever and African swine fever; others became available as the result of controlled game 'cropping' operations. Information on age has been included where adequate observations had been made on the dentition and other biometrical data; otherwise animals were simply regarded as of unknown age, although descriptions such as 'mature', 'immature' or 'young' may have been given.

Most of the sera were separated within 24 hr. of collection and stored, either immediately or after short periods on ice, at $-20°$ C.

Neutralizing antibody detection and titration

Sera were inactivated at 56° C. for 30 min. and mixed with an equal volume of virus diluted to give an estimated $10^{2\cdot0}$ TCD50 per 0·1 ml. The mixtures were placed overnight in the refrigerator (about 4° C.) and inoculated on the following morning, in a dose of 0·2 ml., into primary BK cultures. In screening tests three to five tubes were used per serum sample and the complete protection of one or more cultures by undiluted serum was regarded as evidence of past exposure to Allerton or a closely related virus. This deduction was justified by the fact that frankly negative sera, e.g. from 'insusceptible' species or from uninfected cattle herds, failed to suppress the cytopathic effects of the virus for longer than 2–3 days, whereas sera which had a marginal activity often showed complete protection at this time with virus 'breaking through' later. Occasionally sera which were assessed as positive on screening test were completely negative on titration, although the same stock virus at the same dilution had been employed; again a late virus breakthrough was characteristic of these sera.

Representative samples of positive bovine and game animal sera were titrated for neutralizing activity using four-fold serum dilutions in maintenance medium. The results were expressed as $\log_{10}$ SN50 titres calculated after 5 days of incubation.

RESULTS

Neutralizing antibody to BA virus in East African buffaloes (Syncerus caffer)

Table 1 summarizes the results of screening tests for neutralizing antibody carried out on buffalo sera from N. Tanzania, S. Kenya and W. Uganda. Infection with BA virus was evidently extremely common in these widespread areas, there being only a few young animals in the yearling and 2-year age groups, which were devoid of antibody. In the case of the Serengeti National Park (SNP) it was clear

that infection had existed there since at least late 1967, although no disease episodes attributable to the virus were reported until December 1969. No disease which could be associated with BA virus has ever been reported from the Queen Elizabeth National Park (QENP), Uganda (M. H. Woodford, personal communication).

Table 1. *Neutralizing antibody to BA virus in E. African buffaloes* (Syncerus caffer)

Age group	Serengeti: Tanzania x. 67	Serengeti: Tanzania iii. 69 to viii. 69	Serengeti: Tanzania xii. 69 to ii. 70	Serengeti: Tanzania Total	S. Kenya 1969 and 1970	W. Uganda (Q.E. Park) i. 69 to viii. 69
⩽ 6 months	1/1*	—	3/3	4/4	1/2	1/1
7–18 months	3/3	—	6/6	9/9	0/1	1/3
2–4 years	2/2	3/3	0/2	5/7	0/1	5/5
5–7 years	5/5	5/5	4/4	14/14	—	8/8
⩾ 8 years	7/7	3/3	1/1	11/11	—	43/43
Unknown	—	1/1	—	1/1	3/3 (adults)	—
Totals	18/18	12/12	14/16	44/46	4/7	58/60

* The figures represent no. positive/no. tested.

Table 2. *The titre of neutralizing antibody in Serengeti buffalo sera*

Age group	No. titrated	Mean titre*	Standard deviation	Range
⩽ 6 months	4	1·35	0·58	0·6–1·9
7–18 months	9	1·25	0·57	0·6–2·2
2–4 years	5	1·34	0·50	0·6–1·8
5–7 years	14	1·47	0·26	1·2–1·8
⩽ 8 years	11	1·72	0·35	1·0–2·4
Totals	43	1·46	—	0·6–2·4

* Log_{10} SN 50.

Titrations were carried out on 43 positive buffalo sera from the SNP and the figures obtained are given in Table 2. Titres were, generally speaking, higher than in cattle (*vide infra*) and probably increased with age, as well as showing less variation; the high titres recorded in three of four animals about 6 months old ($10^{1·2}$ to $10^{1·9}$) were almost certainly due to active infection, as mouth lesions were still present in them and passively acquired antibody should have declined to negligible titres at this age (see Rweyemamu, Johnson & Laurillard, 1969, for cattle data).

Kenya buffaloes were found to possess antibodies in three widely separated areas, the titres varying between $10^{1·2}$ and $10^{1·4}$; young animals between 6 months and 2 years of age were devoid of neutralizing antibody in three of four cases.

Neutralizing antibody to BA virus in other wild ungulates

The occurrence of neutralizing antibody in 811 serum samples from 16 species is summarized in Table 3; all sera were derived from areas where infection was shown to occur in cattle or buffaloes. It can be seen that infection with BA virus, or a closely related agent, probably occurs in giraffe (5/33 sera positive); waterbuck

Table 3. *Neutralizing antibody to buffalo Allerton virus in E. African game animals*

Region	Species (common name)	Scientific name	Date of collection	No. positive/ no. tested	Totals
N. Tanzania	Wildebeest	*Connochaetes taurinus*	1965–68	0/113	
Kenya	Wildebeest	*C. taurinus*	1969–70	2/49	2/162
N. Tanzania	Eland	*Taurotragus oryx*	1963–68	0/7	
Kenya	Eland	*T. oryx*	1969–70	1/12	1/19
N. Tanzania	Kongoni	*Alcelaphus buselaphus cokii*	1969	0/1	
Kenya	Kongoni	*A. buselaphus cokii*	1969–70	0/63	0/64
N. Tanzania	Giraffe	*Giraffa camelopardalis*	1969	3/7	
Kenya	Giraffe	*G. camelopardalis*	1969–70	2/26	5/33
N. Tanzania	Impala	*Aepyceros melampus*	1968–70	1/51	
Kenya	Impala	*A. melampus*	1969–70	1/16	2/67
N. Tanzania	Topi	*Damaliscus korrigum*	1969	0/1	
W. Uganda	Topi	*D. korrigum*	1970	0/20	0/21
N. Tanzania	Grant's gazelle	*Gazella granti*	1963–68	0/6	
Kenya	Grant's gazelle	*G. granti*	1969–70	0/14	0/20
N. Tanzania	Thompson's gazelle	*G. thompsonii*	1968	0/35	
Kenya	Thompson's gazelle	*G. thompsonii*	1969–70	0/70	0/105
S. Kenya	Oryx	*Oryx beisa callotis*	1969	2/2	2/3
S. Kenya	Bushbuck	*Tragelaphus scriptus*	1970	0/2	
W. Uganda	Bushbuck	*T. scriptus*	1970	1/6	1/8
S. Kenya	Waterbuck	*Kobus ellipsiprymnus*	1969	3/9	
W. Uganda	Waterbuck	*K. defassa*	1970	1/5	4/14
S. Kenya	Reedbuck	*Redunca redunca*	1969	0/3	
W. Uganda	Reedbuck	*R. redunca*	1970	0/11	0/14
W. Uganda	Uganda kob	*Adenota kob*	1962–70	0/22	0/22
W. Uganda	Hippopotamus	*Hippopotamus amphibius*	1963	20/199	20/199
N. Tanzania	Warthog	*Phacochoerus aethiopicus*	1969–70	0/24	
W. Uganda	Warthog	*P. aethiopicus*	1968–70	0/36	0/60

Table 4. *The distribution of positive sera in hippopotamuses from the Queen Elizabeth National Park, Uganda*, 1963

Age group (years)	No. of animals positive/ no. tested
1–4	1/13
7–8	0/15
10–13	5/43
15–20	1/25
21–25	3/24
28–38	9/78
Total	19/198*

* One positive animal included in Table 3 was of unknown age.

(4/14 sera positive) and hippopotamuses (20/199 positive). There was some evidence for infrequent infection of eland with 1/19 positive; impala (2/67 positive); bushbuck (1/8 positive) and wildebeest (2/163 positive). Two of three oryx also possessed antibody.

The hippopotamuses had been accurately aged (Plowright, Laws & Rampton, 1964) and a breakdown of positive sera according to age groups is given in Table 4; there was no obvious correlation between age and the presence of neutralizing activity. The majority of positive sera were titrated and found to have SN50 end-points in the range $10^{0.5}$ to $10^{1.4}$. Giraffe had low titres, varying from a trace to $10^{0.9}$, waterbuck $10^{0.7}$ to $10^{1.3}$ and one impala $10^{1.5}$.

Neutralizing antibody to BA virus in Tanzanian cattle

Sera from 1098 cattle derived from four to twelve herds in each of six localities situated in the Northern and Lake Provinces of Tanzania (Fig. 1) were screened for the presence of neutralizing antibody to BA virus and the results are shown in Table 5. In July and November 1969 it was apparent that there had already been widespread infection of animals in the 0–6 months and 7–12 months age groups in

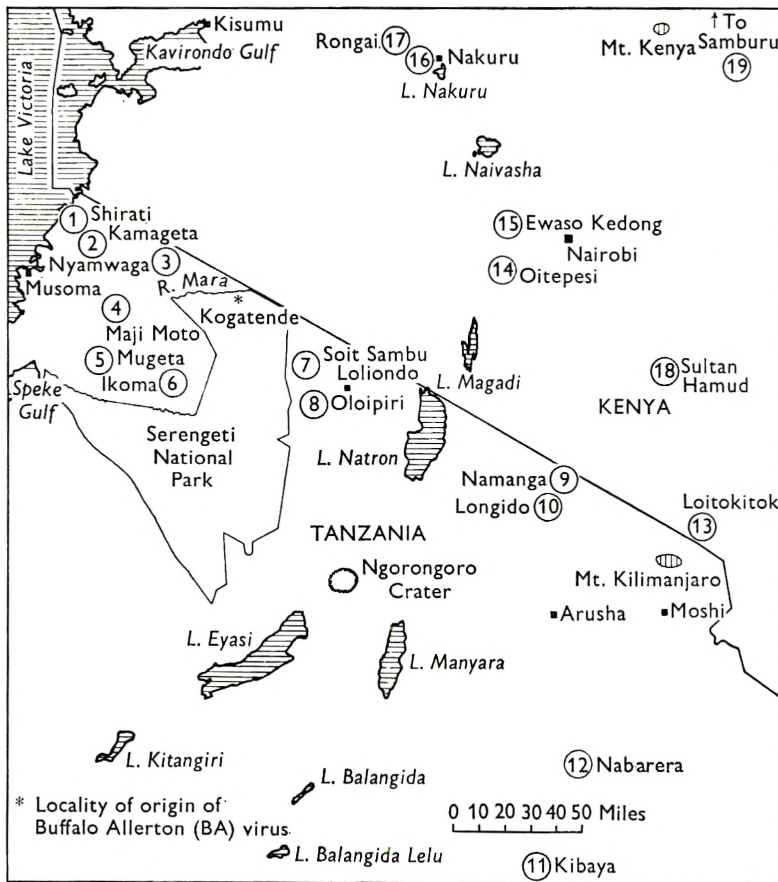

Fig. 1

Table 5. *The distribution of antibody to BA virus in Tanzanian cattle*

Locality*	Date of collection	No. of sera positive/no. tested in age group								Totals
		⩽ 3 mo	4–6 mo	7–12 mo	13–18 mo	17–24 mo	25–42 mo	4–5 yr	⩾ 6 yr	
Shirati (1)	15 xii. 69	0/1	4/17	4/19	3/6	2/2	6/8	2/2	5/5	26/60
Kamageta (2)	18 xii. 69	2/2	3/4	16/24	2/4	4/5	7/7	1/1	3/3	38/50
Nyamwaga (3)	22 xii. 69	1/12	1/5	8/21	4/9	1/1	4/4	2/2	3/4	24/58
Maji Moto (4)	13 xii. 69	1/4	3/7	20/26	23/27	4/4	18/18	2/2	25/25	96/113
Mugeta (5)	3 xxi. 69	5/8	0/15	1/23	3/22	3/9	16/23	4/5	27/28	59/133
Ikoma (6)	8 xii. 69	1/1	2/13	12/23	10/18	7/8	23/27	4/4	19/19	78/113
Totals (Lake Province)		10/28 35·7%	13/61 21·3%	61/136 44·9%	45/86 52·3%	21/29 72·4%	74/87 85·1%	15/16 93·7%	82/84 97·6%	321/527 60·9%
Soit Sambu (7)	xi. 69	26/36		6/7	7/9	1/3	9/10	47/49		96/114
Oloipiri (8)	xi. 69	21/25		20/24	7/7	11/12	25/25	23/24		107/117
Namanga (9)	vii. 69	1/2		11/13	5/7	—	20/21	7/7		44/50
Longido (10)	vii. 69	3/4		17/30	1/3	6/7	8/10	10/11		45/65
Kibaya (11)	i. 70	13/19		12/21	9/11	2/5	9/9	46/50		91/115
Nabarera (12)	i. 70	5/8		8/10	11/15	6/7	19/20	46/50		95/110
Totals (Northern Province)		69/94 73·4%		74/105 70·5%	40/52 76·9%	26/34 76·5%	90/95 94·7%	179/191 98·9%		478/571 83·7%
Kondoa: Singida	vi. 70	6/48		7/60	13/60			16/60		42/228
Kiomboi	vi. 70	4/18		6/59	5/32			18/39		33/148
Totals (Central Province)		10/66 15·1%		13/119 10·9%	18/92 19·6%			34/99 34·3%		75/376 19·9%

* The numbers in brackets are those used in Fig. 1.
N.B. The cattle in localities (1) to (12) above were derived from the herds of 4 to 12 owners.

N. Province; thus more than 70 % of these groups possessed antibody in localities 7–12 inclusive. That the antibody demonstrated was actively acquired is strongly suggested by the considerably lower incidence in cattle of comparable age groups from the Lake Province; thus only 26 and 45 % were positive in the 0–6 months and 7–12 months groups, respectively. The infection rates in adult animals, 4 years or more old, was comparable in both Provinces (97 and 99 % respectively) and hence the proportion of calves acquiring colostral antibody should have been similar.

Table 6. *The titre of neutralizing antibody to BA virus in the serum of Tanzanian cattle*

Age group	No. titrated	Range of titres*	Mean titre*
0– 6 mo	11	0·4–1·6	0·90
7–12 mo	13	0·5–1·8	0·77
13–18 mo	7	0·4–1·1	0·64
19–24 mo	14	0·5–1·2	0·69
25–42 mo	11	0·2–1·2	0·74
4–5 yr	8	0·4–1·2	0·64
$\geqslant$ 6 yr	27	0·4–1·8	0·89

* Log_{10} SN 50.

In the Lake Province there were clear indications that the infection during December had reached various stages of completion in different localities. Thus at Mugeta (5) only 4 of 60 and at Shirati (1) only 11 of 42 animals between the ages of 4 and 18 months possessed antibody; the corresponding figures for Kamageta (2) and Maji Moto (4) were 21 of 32 and 46 of 60 respectively. As the proportion of positive cattle in age groups of 2 years or more was consistently very high it appeared reasonable to assume that BA virus infection was passing through the younger cattle of this extensive Province during December 1969, at the same time as buffaloes in the Kogatende area (Fig. 1) were suffering from a mortality in which this agent was involved.

Sera collected in June 1970 in the Kondoa: Singida area, immediately to the south of the zone already described, showed a very much lower rate of infection with only 34 % of positives in the $\geqslant$ 25 months age group (Table 5). In August 1970 a further 527 from 2 to 3 years old cattle in the same area were examined and only 85 (16·1 %) possessed antibody.

A total of 91 positive cattle sera from the Lake Province, collected during December 1969, were titrated for antibody to BA virus, the results being given in Table 6. It is probable that much of the antibody in calves up to 6 months of age, including six which were aged 3 months or less, was passively acquired; the mean titre was approximately the same as for older cows ($\geqslant$ 6 years), whereas intermediate groups showed a moderate decline. The higher titres in older cattle suggested that they may have resulted from frequent reinfection or perhaps chronic infection, although there is as yet no firm evidence for either of these suggestions. The pattern was similar to that in buffaloes (Table 2) but the mean titres recorded were consistently lower.

Neutralizing antibody in sheep and goats in Tanzania

Between December 1967 and February 1968 sera were collected from sheep and goats of various ages in the Loliondo District of N. Tanzania (Fig. 1).

There was only one positive sample among 44 sheep sera and one more among 148 goat sera; the titres of these were $10^{0\cdot7}$ and $10^{1\cdot1}$ respectively. If a comparison is made with cattle in the same district (localities 7 and 8, Table 5) it is evident that small domestic ruminants are not important hosts of Allerton-type virus, even when exposed to heavy infection. Capstick (1959) found that Allerton-type virus caused reactions to high titre when inoculated intradermally in these species and a Kenya strain induced local skin lesions on inoculation of sheep, followed by the development of neutralizing antibody (MacOwan, 1962).

Neutralizing antibody in cattle in Kenya and Uganda

In the Masailands (Rift Valley Province) of S. Kenya infection with Allerton-type virus was approximately as frequent as in the W. Province of Tanzania. Thus 85% of cattle which were ⩾ 25 months of age possessed antibody, the proportion declining to 30% in the first 6 months of life with evidence of active infection in the two intermediate groups (Table 7).

Table 7. *The distribution of antibody to BA virus in Kenyan cattle*

Locality*	Date of collection	No. positive/no. tested in age group: 0–6 mo	7–12 mo	13–24 mo	⩾ 25 mo	
Loitokitok (13)	i. 70	0/6	5/16	4/13	7/11	16/46
Oitepesi (14)	i. 70	5/26	17/33	9/19	14/19	45/97
Ewaso Kedong (15)	i. 70	10/20	15/26	15/20	40/44	80/110
Sultan Hamud (18)	ii. 70	3/7	21/29	5/8	24/26	53/70
Total for Rift Valley		18/59	58/104	33/60	85/100	194/323
Province		30·5%	55·8%	55·0%	85·0%	60·1%
Nakuru: Rongai	vii./xi.	—	—	48/70	—	48/70
(16:17) A†	1969	—	—	68·6%	—	68·6%
Nakuru: Rongai	vii. 69–	—	—	0/289	—	0/289
(16:17) B‡	viii. 70	—	—	—	—	0·0%
Samburu (19)	vii. 70	16/32	19/24	10/14	35/40	80/110
		50·0%	79·2%	71·4%	87·5%	72·7%

* The numbers in brackets are those used in Fig. 1.
† Sum of two positive groups bought from cattle dealer.
‡ Sum of eight negative groups bought from cattle dealer.

In the Samburu District of N. Kenya, cattle owned by a semi-nomadic pastoral people related to the Masai showed a very high rate of infection with 87·5% of cattle positive in the age group ⩾ 25 months (Table 7).

In the central, highly developed, part of the Rift Valley Province of Kenya (Nakuru–Rongai in Fig. 1) the incidence of infection was estimated by the examination of sera from groups of experimental cattle, about 18–24 months old, which were collected by a dealer over a period of about 1 year. Eight of ten groups were

completely negative and the other two showed a high proportion of positives (69 %), comparable to that in less-developed regions (see Table 7). It was not possible to trace the farms of origin of any of these cattle but the results were highly suggestive of an irregular distribution of virus.

In Uganda, relatively small numbers of samples from the northern provinces were examined. There was a considerably higher incidence of infection in the Karamoja and Teso districts, where infection rates were comparable to those in the western Lake Province of Tanzania (Table 8). In the W. Nile district, however, the infection rate was lower, not exceeding 43 % and not apparently increasing materially after the age of 7–12 months.

Table 8. *The distribution of antibody to BA virus in Ugandan cattle*

	No. of sera positive/no. tested in age group				
Province	0–6 mo	7–12 mo	13–24 mo	⩾ 25 mo	Totals
Karamoja	—	1/1	26/38	100/111	126/149
	—	—	71·0%	83·0%	80·0%
Teso	4/4	7/10	7/10	105/128	123/152
		70·0%	70·0%	82·0%	80·9%
W. Nile	0/1	2/8	24/53	16/48	42/109
	—	25·0%	43·0%	32·0%	37·4%

DISCUSSION

The seriological results presented in this paper show that infection with Allerton-type herpes virus occurs regularly in all the E. African buffalo populations which were sampled; every animal estimated to be more than 2 years of age possessed neutralizing antibody and a large proportion had probably been infected by the age of 18 months. In spite of the presence of distinctive ulcerations in the mouth of sick buffaloes in the 1969 incident (Schiemann *et al.*, to be published) no other indications have been reported that the virus causes overt disease in buffaloes. This is particularly significant in the case of the population in the Queen Elizabeth National Park, Uganda, where the species was under close veterinary observation during the period when sera were being collected (M. H. Woodford, personal communication). It therefore seemed to be highly unlikely that the virus was a primary cause of the severe morbidity and mortality observed in 1969 in the Serengeti region of Tanzania (Schiemann *et al.*, to be published).

The occurrence of significant titres of neutralizing antibody in a smaller proportion of some other game animal species, exposed to more or less close contact with buffaloes or cattle, suggests that the same or a related virus may infect giraffe, oryx, waterbuck and the hippopotamus, possibly also infrequently eland, impala and wildebeest. It could be that some of these species are not susceptible to infection with Allerton virus but are commonly infected with similar herpes viruses, which only induce cross-neutralizing antibody in a few individuals.

There is no doubt that, among primates, several herpes viruses occur in Old World monkeys, including B virus (Keeble, Christofinis & Wood, 1958); SA8

(Malherbe & Harwin, 1958; Malherbe, Harwin & Ulrich, 1963); a cytomegalovirus (Black, Hartley & Rowe, 1963) and LV virus (Clarkson, Thorpe & McCarthy, 1967). In New World monkeys and marmosets another agent, herpes-T or marmoset herpes virus, has been shown to cause severe, generalized infection in some species but an inapparent or mild infection in others (Holmes, Devine, Nowkowski & Deinhardt, 1966; Daniel *et al.* 1967). It seems highly probable that many herpes viruses, sometimes closely related antigenically, will eventually be found in the richly varied ungulate fauna of Africa; one of these, the cause of malignant catarrhal fever in cattle, has already been shown to be universal in E. African wildebeest populations (Plowright, 1965; 1967) and neutralizing antibody to this or a closely related agent is also present in two other members (topi and kongoni) of the same family, the Alcelaphinae (Plowright, unpublished work).

The apparent limitation of lesions caused by BA virus to the upper alimentary tract of buffaloes (Schiemann *et al.*, to be published) is in distinct contrast to the original association of this virus in cattle in South Africa, Ruanda Urundi and Kenya with a mild form of 'lumpy skin disease' (Alexander *et al.* 1957; Huygelen, Thienpont & Vandervelden, 1960; MacOwan, 1962). Huygelen *et al.* (1960) did, however, mention the occurrence of erosions of the buccal mucosa and described the transmission of infection by scarification of the tongue mucosa of experimental cattle. Such lesions have not been observed by other investigators whether in Africa (Capstick, 1959; Weiss, 1963) or in Britain (Lepper, Haig & Wilcox, 1969; Martin *et al.* 1969).

The high rate of infection in E. African cattle was not entirely unexpected, since the examination of limited numbers of sera had already shown that infection with Allerton-type virus was not uncommon in Kenya and South Africa (Weiss, 1963; Martin & Gwynne, 1968). Figures provided by the latter authors were conservative in that sera were only screened at a dilution of 1/4, a procedure which would almost certainly have missed many animals with previous exposure to the virus. Their figures, like our own and those of Rweyemamu *et al.* 1969, show a definite tendency for antibody to increase in frequency with age. Nevertheless, it could hardly have been foreseen that about 85–95 % of cattle more than 2 years of age would show evidence of past infection in vast areas of E. Africa including the Masailands, Sukumaland (Lake Province, Tanzania), Samburu and Karamoja, and that the infection was never absent in any locality tested.

All the areas of higher incidence are characterized by big cattle populations, with semi-nomadic pastoralists owning large herds which are frequently brought to communal watering points and herded at night into crowded enclosures for protection against predators. All of them possess, incidentally, relatively large herds of buffaloes but there is no reason, at present, to suppose that this species is essential for the initiation or maintenance of infection, though they may undoubtedly contribute to the latter. The widespread inapparent infection of buffaloes probably means that Allerton-type virus has been present in this species for a very long time and is not a relatively recent introduction to Africa as suggested by Rweyemamu (1969).

Outside the areas of very high incidence, as in the Central Province of Tanzania

and the W. Nile District of Uganda, there was some evidence, as also in the Nakuru–Rongai area of Kenya, that the frequency of antibody varied considerably from herd to herd, some showing a moderate to high infection rate, others a very low or nil rate. This possibly reflects infrequent inter-herd contacts and a more static animal husbandry with smaller individual holdings.

The variable antibody rates in young cattle sampled during December 1969 in different localities of the Lake Province of Tanzania suggests that a wave of infection was passing through the area at that time and raises the question of the usual method of transmission of the virus. Weiss (1963) reported that, when animals infected with Allerton virus were kept in close contact with susceptible cattle, in an insect-proof stable, transmission did not occur even when attempts were made to favour virus transfer by handling susceptible animals after sick ones. In this laboratory we have also failed on several occasions to demonstrate contact infection of susceptible cattle which were housed together with excretor animals and drank from the same water-bowls. It seems possible that a flying vector is involved since Allerton virus was isolated from *Biomyia fasciata* caught on infected cattle and persisted for 6 days in flies which had fed on infected cell-culture fluids; transmission experiments using infected flies failed, however (du Toit & Weiss, 1960). No observations on possible vectors were made in the present instance but ticks and lice were observed in large numbers on sick buffaloes (Schiemann *et al.*, to be published). Rweyemamu *et al.* (1969) have suggested that biting flies may be involved in Britain in the transmission of bovine herpes mammillitis virus (BHMV), which is immunologically indistinguishable from Allerton virus (Martin, Hay, Crawford, le Bouvier & Crawford, 1966).

No clinical signs attributable to Allerton virus have been reported in E. African cattle in the areas of highest incidence. It remains to be determined whether the usual manifestation is a necrotic and ulcerative stomatitis, as described in sick buffaloes. In this connexion it is interesting to note that Lepper *et al.* (1969) and Martin *et al.* (1969) noted lesions of the rhinarium and nostrils, similar to those produced by us in experimental cattle with BA virus inoculated intravenously (Kalunda & Plowright, to be published). Such necrotic and eroded areas would not by themselves be expected to attract attention in most parts of E. Africa. There is no evidence that lesions comparable to those caused by BHMV in Britain occur in E. African cattle; Martin & Gwynne (1968) failed to detect such cases in Kenya and the vast majority of serological conversions occur before breeding age (Tables 5 and 7).

The cumulative evidence lends considerable support to the hypothesis, first put forward by Martin, Martin, Hay & Lauder (1966) that Allerton virus, *alias* BHMV, behaves epidemiologically in *Bovinae* like herpes simplex in man. Thus it is capable of causing a number of clinical syndromes, including generalized pseudo-lumpy skin disease, mammillitis and gangrene of the skin of the udder (Martin, Martin & Lauder, 1964; Rweyemamu, Johnson & Tutt, 1966; Derbyshire & Haig, 1969) and necrotic and ulcerative lesions of the upper alimentary and respiratory tracts. It has not, so far, been reported as causing genital lesions or systemic infections of the newborn but these are obvious possibilities. In the meantime, is there any real

justification for adopting different names for indistinguishable strains of virus derived from diverse clinical syndromes?

The demonstration of a primary association of some strains of Allerton virus with the oral cavity of cattle would provide a very close analogy with herpes simplex in man or with some of the simian viruses such as herpes B or T, both of which naturally cause ulcerative lesions of the mouth mucosae (Keeble *et al.* 1958; Daniel *et al.* 1967). Allerton virus and the primate agents mentioned all fall into the subgroup A of Melnick *et al.* (1964) characterized by ready release from cultured cells and they all produce large syncytia in monolayers. A surprising feature for a herpes virus would be the common involvement of an insect vector but contact transmission of herpes T virus from sick squirrel monkeys to marmosets has already been shown to occur with difficulty if at all (Daniel *et al.* 1967); perhaps biting flies would also be able to effect virus transmission in this case.

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Further studies on the growth of rubella virus in human embryonic organ cultures: preliminary observations on interferon production in these cultures

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SUMMARY

Organ cultures prepared from 15 different organs obtained from 43 fetuses were consistently found to support the growth of rubella virus, irrespective of the gestational age of the fetus or the strain of rubella virus inoculated. Although rubella virus replicated in fetal lenses, adult lenses did not support the growth of rubella virus. Organs obtained from four fetuses between 8 and 17 weeks gestational age produced similar titres of an inhibitor which had the characteristics of interferon. The use of Trowell T8 medium and incubation in a mixture of 5 % CO_2 in oxygen provided the most suitable conditions for the maintenance of most organ cultures. Under these circumstances it was possible to obtain adequate histological preparations from these organs, but light microscopy studies revealed no significant differences in sections of rubella inoculated and control organ cultures.

INTRODUCTION

Many viruses which cause acute repiratory infections can be cultivated in human embryonic organ cultures, particularly trachea and nasal epithelium (Hoorn & Tyrrell, 1965). This system provides a convenient method for determining the properties of some viruses which can only be cultivated with difficulty in monolayer cell cultures (Tyrrell & Bynoe, 1965; Almeida & Tyrrell, 1967). We previously established that rubella virus could be propagated in embryonic organ cultures derived from the upper respiratory tract (Best, Banatvala & Moore, 1968) and showed that virus could be detected electron microscopically in ultra-thin sections prepared from these cultures (Kistler, Best, Banatvala & Töndury, 1967). This communication describes our investigations on the growth of rubella virus in numerous other organ cultures derived from fetuses of varying gestational age and includes preliminary studies on the production of interferon in organ cultures derived from four of these fetuses. In addition, we have investigated different conditions for maintaining various organ cultures in a satisfactory condition for histological studies.

MATERIALS AND METHODS

Organ cultures

Organs were obtained within 1–22 hr. of hysterotomy from 'healthy' human fetuses varying in age from 8 to 28 weeks gestation. Fifteen different organs were used in these studies: brain (cerebrum), heart, kidneys, lens, liver, lung, nasal epithelium, trachea, pharynx, larynx, retina, skin, spleen, adrenal and chorion. Considerable care was taken in the preparation of organ cultures since we were interested not only in producing the best possible conditions for histological studies by light microscopy but also in preserving the cellular ultrastructure in a satisfactory condition for electron microscopic studies, details of which will be published later. Organs were cut into pieces no larger than 2 $mm.^3$ using new razor blades which were discarded after three cuts. Fragments were handled with strips of filter paper in order to reduce mechanical damage to a minimum. The virus inoculum was allowed to adsorb for approximately 2 hr. at room temperature or 4° C. and was then replaced with sufficient medium to ensure that all the fragments in the dish were completely covered. The medium was changed every 1–2 days. Control cultures were inoculated with cell culture fluid from non-infected cell cultures.

Growth of rubella virus in fetal lenses was compared with that in adult lenses which were obtained from eyes removed as soon as possible after death.

The following media were used for the maintenance of organ cultures:

(1) Medium 199 (Wellcome Reagents) containing 0·088 % sodium bicarbonate and either 0·2 % bovine plasma albumin or fetal calf serum ranging between 2 and 50 %.

(2) Medium Trowell T8 (Difco Laboratories) containing 10 % fetal calf serum (Trowell, 1959).

(3) Leibovitz L15 medium (Flow Laboratories Ltd.) containing 2 % fetal calf serum and 0·029 % glutamine. This medium enabled cultures to be incubated in air without added CO_2.

(4) Since the aqueous humour of the adult lens contains a high concentration of ascorbic acid, medium NCTC 135 (Flow Laboratories Ltd.), a medium which contains a high concentration (49·9 mg./l.) of ascorbic acid, was used for the maintenance of lenses with the addition of 50 % fetal calf serum.

All media contained 200 units/ml. penicillin and 200 μg./ml. streptomycin.

Cultures were incubated at 36° C. in an atmosphere of 5 % CO_2 in air or 5 % CO_2 in oxygen.

Inoculation of rubella virus

Four strains of rubella virus were used for inoculation of organ cultures. Strain Judith, a well-adapted laboratory strain (McCarthy & Taylor-Robinson, 1965) was propagated in RK-13 and BHK-21 cell cultures. Strains Giguere and Thomas were isolated from infants with congenital rubella in 1964 and 1966 respectively, while Portsmouth was isolated in 1967 from a child with postnatally acquired

rubella. These three strains had been passed less than 10 times in RK-13 and BHK-21 cell cultures.

Organ cultures were inoculated either on the day of preparation or 1–2 days later. Virus samples used for inoculation varied in titre from 10 to $10^{7\cdot5}$ TCD50/ml. Although organ cultures could be infected by inoculating as little as 0·2 ml. rubella virus, in most experiments the virus inoculum was sufficient to cover the organ fragments. The inoculum was allowed to adsorb at room temperature or 4° C. for periods varying from 2 to 8 hr., after which it was replaced with fresh medium.

Control cultures were included in each experiment and were either left uninoculated or were inoculated with an equal volume of fluid from non-infected cell cultures. In order to test the survival time of the inoculated virus, a virus control dish containing virus but no organ culture was included in some experiments.

Virus titrations

Organ culture fluids were harvested at intervals, 'snap' frozen and stored at −70° C. until they could be titrated in parallel in RK-13 cell cultures (Best & Banatvala, 1967).

Interferon assay

In order to determine whether infection by rubella virus resulted in interferon production, fluids from rubella inoculated cultures prepared from four different fetuses were harvested daily for up to 5 days. A sample was taken from each harvest for virus titration and the remainder was adjusted to pH 2 by dialysis against glycine buffer pH 2 for 48 hr., followed by dialysis against phosphate buffered saline (PBS), pH 7·2 for 24 hr. to restore the pH to neutrality. Interferon assays were carried out in cultures of a continuous line of vervet monkey kidney, V3A (K. H. Fantes, personal communication). Cultures were challenged with 100 TCD50/ml. Sindbis virus 18 hr. after the inoculation of twofold dilutions of organ culture fluid and results were recorded when the Sindbis controls showed 75–100% cytopathic effect. The titre of interferon was taken as that dilution giving 50% inhibition of cytopathic effect. A standard human interferon sample of known titre was included in each test.

Histological studies

Organ fragments were fixed in Bouin's fluid and embedded in paraffin wax for sectioning. Preparations were stained with haematoxylin and eosin.

RESULTS

Maintenance of organ cultures

Although trachea and nasal epithelium could be maintained in a satisfactory condition for at least 34 days in medium 199 containing 0·2% bovine plasma albumin or 2% fetal calf serum in an atmosphere of 5% CO_2 in air, most other organs became rather friable after 2–3 days incubation in this medium, although degeneration did not occur so quickly when higher concentrations (10–50%) of fetal calf serum were used.

Table 1. *The effect of fetal age on rubella virus titres*

Gestational age of fetus (weeks)	Highest rubella virus titre detected in the organ culture fluid*												
	Cerebrum	Heart	Kidney	Lens	Liver	Lung	Nasal epithelium	Pharynx	Retina	Skin	Spleen	Suprarenal	Trachea
8	1·5†	—	1·5	—	—	< 1·5	—	—	—	—	—	—	—
11	—	—	2·8	—	—	2·0	—	—	—	—	—	—	2·5
11½	—	—	3·5	2·3	—	3·5	—	—	—	—	2·5	—	2·2
12	1·8	< 1·5	3·2	—	1·5	2·0	—	—	—	—	—	—	2·5
12	—	—	2·7	2·5	3·0	2·5	—	—	—	—	2·2	—	—
14	—	2·5	—	1·8	—	2·5	—	—	—	—	—	—	—
15	—	—	—	—	—	—	—	—	—	—	—	—	3·5
15	2·2	2·5	—	2·2	2·5	2·6	—	—	2·0	2·5	2·0	—	2·8
15	—	—	1·5	—	—	—	—	—	—	—	1·4	—	—
15	—	—	—	2·3	—	—	—	—	—	—	—	—	—
15½	—	1·5	1·6	1·5	—	1·3	—	—	—	1·5	2·5	—	< 1·0
16	—	—	—	—	—	—	3·3	—	—	—	—	—	2·4
16	—	—	—	—	—	—	1·5	—	—	—	—	—	1·0
16	—	1·8	1·3	—	—	2·5	—	—	—	< 1·5	—	1·5	—
16½	2·0	—	> 2·5	2·0	2·0	2·1	2·5	—	—	2·0	1·5	—	—
17	—	—	—	—	—	—	—	—	—	—	3·8	—	2·2
17	—	3·0	—	2·2	—	2·5	—	—	1·6	—	2·6	—	—
18	2·0	—	—	—	—	—	4·0	—	—	—	—	—	2·8
19	2·5	—	—	—	—	2·5	—	—	—	—	—	—	—
19	—	—	2·3	—	—	—	—	—	—	—	—	—	—
23	—	—	—	—	—	—	3·7	1·5	—	2·5	—	—	2·5

* Organ cultures were inoculated with 10^{5}–$10^{7\cdot5}$ TCD 50/ml. rubella virus. † $\log_{10}$ TCD 50/ml.

Use of Leibovitz L15 medium had the advantage that a CO_2 incubator was not necessary for incubation of cultures. Although cultures maintained in this medium continued to produce virus for at least 12 days they did not maintain in satisfactory condition for detailed histological studies.

Medium Trowell T8 containing at least 10% fetal calf serum and incubation in an atmosphere of 5% CO_2 in oxygen provided the most suitable conditions for the maintenance of organ cultures, since most organs remained in good histological condition for at least 7 days in this medium. However, both fetal and adult lenses became opaque in this medium but when they were incubated in medium NCTC 135 containing 50% fetal calf serum in an atmosphere of 5% CO_2 in air they could be maintained for at least 13 days without becoming opaque. Cultures of retina were also incubated in 5% CO_2 in air since it has been reported that high concentrations of oxygen result in oxygen poisoning of the retina (Lucas & Trowell, 1958).

Growth of rubella virus in different organs

Organ cultures prepared from 43 different fetuses obtained between 8 and 28 weeks gestation supported the growth of rubella virus when inoculated with $\geqslant$ 10 TCD50/ml. No rubella virus was recovered in one experiment in which tracheal and spleen organ cultures prepared from a 14½-week fetus were inoculated with 10^5 TCD50/ml. strain Thomas.

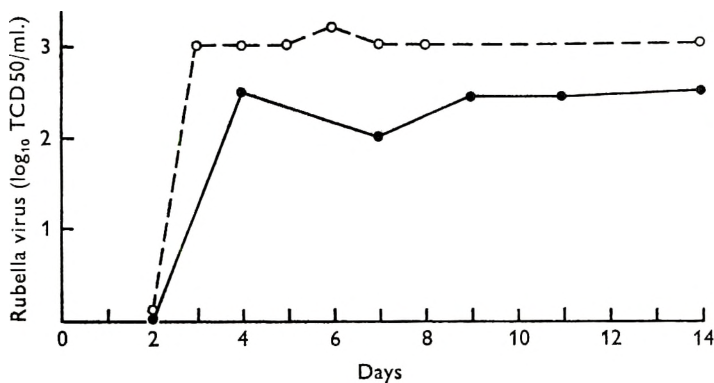

Fig. 1. Two typical growth curves showing the multiplication of rubella virus in organ cultures of spleen from two fetuses aged 16 and 17 weeks.

All 15 organs so far tested supported the growth of rubella virus. Table 1 shows that titres of between $10^{1\cdot5}$ and 10^3 TCD50/ml. were usually obtained irrespective of the gestational age of the fetus. Experiments in which a virus inoculum of $< 10^5$ TCD50/ml. was used are not included in this table. The well-adapted laboratory strain Judith was employed in the majority of experiments but when Portsmouth, Giguere and Thomas, the low passage strains, were used similar titres were obtained. The highest titre obtained was 10^4 TCD50/ml. from cultures of nasal epithelium from an 18-week fetus. The variations in the titre of rubella virus recovered may have been influenced by variation in size of organ fragments

placed in each dish. Some low titres may also be explained by the fact that although some cultures appeared macroscopically to be in good condition, when examined microscopically they were found to have undergone considerable degeneration.

Typical growth curves obtained from organ cultures of spleen are illustrated in Fig. 1. The titre of virus in the surrounding fluid was $10^{2\cdot5} - 10^3$ TCD50/ml. on day 3 and was maintained at approximately this level until experiments were discontinued on day 14. Most organs continued to produce similar titres of virus as long as the organ fragments remained in a healthy condition. Since it was found that low titres of rubella virus could be recovered from virus control dishes inoculated with approximately 10^6 TCD50/ml. for 2 days after inoculation, organ culture fluids from days 1 and 2 were not titrated. Results of a typical experiment using fragments of 9 different organs obtained from a 15-week fetus and maintained in medium Trowell T8 are shown in Table 2.

Table 2. *Rubella infected organ cultures from fetus aged* 15 *weeks.* 10^6 *TCD50/ml. virus (Judith) inoculated*

	Infected cultures Titre ($\log_{10}$ TCD 50/ml.)		
Organ	Day 4	Day 7	Condition of inoculated and control cultures
Trachea	2·8	2·2	Good
Lung	2·6	2·6	Good
Liver	2·5	2·0	Good
Cerebrum	2·2	1·5	Slightly friable
Spleen	2·0	1·6	Good
Heart	2·5	2·5	Good
Skin	2·6	2·5	Good
Lens	2·2	1·8	Clear
Retina	1·0	2·0	Good

No virus was recovered from control cultures.

Growth of rubella virus in the lens

Although rubella virus was found to multiply consistently in 13 lenses obtained from fetuses of 11½ to 19 weeks gestation, no rubella virus was recovered from five adult lenses inoculated and maintained under identical conditions.

Interferon production

All organs tested from four fetuses obtained between 8 and 17 weeks gestation produced an interferon-like inhibitor in the organ culture fluid which was detected within 24 hr. of inoculating rubella virus. However, by day 3, when rubella virus titres began to increase, the amount of inhibitor decreased, being generally undetectable 4 days after virus inoculation (Table 3). No inhibitor was detected in control cultures. Increasing gestational age did not appear to be associated with an increased capacity to produce interferon; the amount of interferon present in organ culture fluid in response to infection by rubella virus was low in all four fetuses studied ($\leqslant$ 8 units/ml.).

This inhibitor had the following characteristics which strongly suggested that it was interferon:

(1) It was non-dialysable.

(2) It was stable at pH 2.

(3) Its activity was destroyed by treatment with 0·1 % trypsin.

(4) It was inactivated by heating for 1 hr. at 56° C.

(5) It was not sedimented by centrifugation at 100,000 *g* for 1 hr.

(6) The inhibitor was not neutralized when a sample was incubated with rubella antiserum, thus excluding the possibility that the inhibitory effect was due to interference by residual rubella virus.

(7) It appeared to be species specific since it did not inhibit the growth of Sindbis virus in chick embryo fibroblasts.

Table 3. *Production of interferon by organ cultures*

Organ culture	Age of foetus in weeks	Inoculum	Interferon titre, units/ml. Day 1	2	3	4	5
Cerebrum	8	Judith	4	2	2	—	—
Kidney	—	10^7 TCD 50/ml.	4	3	< 2	—	—
Lung	—		4	4	3	—	—
		Control cultures	< 2	—	—	—	—
Spleen	16	Judith	8*	—	—	—	—
		$10^{4\cdot3}$ TCD 50/ml.	—	—	—	—	—
		Control cultures	< 2*	—	—	—	—
Cerebrum	16½	Judith	4	8	8	< 2	—
Kidney		10^5 TCD 50/ml.	8	8	4	< 2	< 2
Lens			4	< 2	< 2	< 2	—
Liver			< 2	8	< 2	< 2	—
Lung			< 2	4	< 2	< 2	—
Nasal epithelium			< 2	8	4	< 2	—
Skin			4	4	2	< 2	< 2
Spleen			8	4	< 2	< 2	< 2
		Control cultures	< 2	< 2	—	—	—
Spleen	17	Judith	4 (8)†	—	—	—	—
		10^6 TCD 50/ml.	< 4 (8)	—	—	—	—
		Control cultures	< 4	—	—	—	—

* Harvests from days 1, 2 and 3 were pooled.

† Figures in parentheses show the intracellular interferon titre. Organ fragments were ground up and an approximate 4 % suspension prepared. After centrifugation at 2000 rev./min. for 10 min. the supernatant was tested for interferon.

Histological studies

Using medium 199 and Leibovitz L15 medium the condition of all organs except trachea and nasal epithelium was generally too poor for detailed histological examination, but considerable improvement was seen when smaller (< 2 mm.³) pieces of each organ were maintained in T8 medium containing 10 % fetal calf serum in an atmosphere of 5 % CO_2 in oxygen, provided the medium was changed frequently (Table 2). When organ culture fragments maintained in this medium

were examined 7 days after inoculation no gross histological changes or inclusion bodies were observed, although rubella virus was present at titres up to 10^4 TCD50/ml. in the surrounding fluid. Extensive vacuolation and capsular hyperplasia were seen in some rubella virus-infected fetal lenses, but these changes may have been artefacts since similar but less extensive effects were noted in some controls. Satisfactory sections could not be obtained from adult lenses, which were hard and difficult to cut.

DISCUSSION

In previous studies employing organ cultures of trachea and nasal epithelium, we found that both control and rubella virus-infected cultures could be maintained for at least 34 days without loss of ciliary activity. Histological examination showed that the epithelial surface was intact and cilia were present on both control and rubella virus-infected cultures. No degenerative changes, ulceration or inclusion bodies were present (Best, Banatvala & Moore, 1968).

Studies reported in this paper show that many other human embryonic organ cultures will support virus multiplication and may be maintained in a suitable condition for histological examination provided care is taken to ensure that small fragments are used and that these are maintained in a specialized medium, such as T8. Trowell (1959) originally recommended this synthetic medium for maintaining cultures derived from different organs of adult rats. Although it contains fewer total constituents than medium 199 this medium incorporates thymine and extra cysteine. Because the concentration of oxygen is likely to be at its lowest in the centre of organ fragments it is important to ensure that fragments are no larger than 2 $mm.^3$ in size and since the solubility of oxygen in biological media is low, Trowell recommended the use of 5% CO_2 in oxygen rather than in air in order to achieve higher oxygen concentrations.

Although no histological changes were evident, rubella virus replicated consistently in fetal but not in adult lenses. That adult lenses were refractory to infection is consistent with the finding that lens lesions have not been reported following postnatally acquired infection. This may be because rubella virus cannot penetrate or infect the adult lens capsule or perhaps because adult lens cells will not themselves support the growth of rubella virus. We previously demonstrated rubella virus particles and osmiophilic inclusion bodies containing vesicular structures and 'myelin whorls' in ultra-thin sections of human embryonic trachea and nasal epithelium (Kistler *et al.* 1967). Preparations of all other organs except lens were satifactory for electron microscopy when they had been maintained in medium Trowell T8 containing at least 10% fetal calf serum. Lenses could be maintained in a satisfactory condition in medium NCTC 135 containing 50% fetal calf serum (Kistler, Best & Banatvala, unpublished observations).

Our experiments have shown that rubella virus replicates consistently *in vitro* in human embryonic organs of widely differing gestational age. This is consistent with recent reports that, after laboratory confirmed maternal rubella during the first trimester, if sensitive techniques for virus detection are used, rubella virus can be recovered from 92 to 95% of fetuses (Rawls, Desmyter & Melnick, 1968;

Thompson & Tobin, 1970) and also with reports that even if acquired after this time fetal infection may occur. However, in such cases developmental anomalies are usually more subtle than if infection is acquired earlier and may not be detected unless infants are followed-up for a prolonged period (Hardy, McCracken, Gilkeson & Sever, 1969). Even though maternal infection during the first trimester almost invariably results in fetal infection, it is unlikely that, if allowed to proceed to term, all infected fetuses would be affected adversely. Thus, the fetus may be capable of limiting or terminating infection and it has been suggested that interferon may be involved in this process (Mims, 1968; Rawls, 1968). However, there have been few studies on human fetal interferon production, although it has been shown that young chick and mouse embryo cells produce less interferon than older ones (Isaacs & Baron, 1960; Sawicki, 1961; Heineberg, Gold & Robbins, 1964). Our studies suggest that the fetus is capable of producing from an early gestational age a substance which has the physical properties of interferon, but this response does not increase as the fetus matures. The time sequence of its development closely paralleled that of interferon induced in calf trachea organ cultures infected with para-influenza and rhinoviruses (Smorodintsev, 1968).

Cantell, Strander, Saxen & Meyer, (1968) studied interferon responses of human lymphocytes obtained from fetuses, newborns, children and adults infected with Sendai virus and also demonstrated that, although competent at an early gestational age, the amount of interferon produced was relatively constant during intrauterine as well as postnatal life. In our studies only low interferon titres were produced by rubella virus, but it is likely that rubella is a poor interferon inducer in fetal cells, this being supported by our finding that in comparison to such viruses as the para-influenza viruses and some rhinoviruses rubella produces only small amounts of interferon in human embryonic lymphocytes (Banatvala & Bowern, unpublished observations). A persistent fetal infection frequently follows maternal infection during the first trimester. Although this may perhaps be in part because rubella virus is a relatively poor interferon inducer, Siewers, John & Medearis (1970) showed that young human fetal fibroblastic cell lines between the 10th and 50th passages infected with Sindbis and vesicular stomatitis viruses were less sensitive to the action of interferon than cells derived from older fetuses. We hope to determine the interferon sensitivity of primary fetal cell cultures of varying gestational age infected with rubella virus.

Our investigations have shown that human embryonic organ cultures provide a useful system for studying some aspects of the pathogenesis of fetal infection by rubella. They may provide a useful experimental system for further studies on the distribution and spread of virus in different organs and also its susceptibility to inhibitory and chemotherapeutic agents. Furthermore, for studies on the pathogenesis of infection, cells maintained as organ cultures have the additional advantage that, unlike those in monolayer cell cultures, they are similar in structure and physiology to those in the intact human host.

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The evaluation of a live salmonella vaccine in mice and chickens

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SUMMARY

Mice vaccinated with a live attenuated strain of *Salmonella dublin* were protected against heavy challenge infections of *S. dublin*, *S. typhimurium*, *S. choleraesuis* and *S. anatum*. Oral and subcutaneous vaccination were equally effective. When day-old chicks were orally vaccinated and subsequently challenged with *S. typhimurium*, the growth of the challenge organism was considerably reduced or eliminated from the livers of the vaccinated chicks whereas most of the non-vaccinated were heavily infected. Field trials with vaccinated day-old chicks showed that they suffered no setbacks in growth, stress, loss of appetite or adverse side effects.

INTRODUCTION

Many attempts have been made to control salmonellosis in animals with killed vaccines, but any protection was short-lived and ineffective against heavy infections. Specific live vaccines have been produced to protect chickens against *S. gallinarum* (Smith, 1956*a*), pigs against *S. choleraesuis* and calves against *S. dublin* (Smith, 1965); Rankin, Newman & Taylor (1966) showed that a live *S. dublin* vaccine (strain 51, Smith, 1965) would protect calves against *S. typhimurium*. Our findings show that this vaccine protects mice also against salmonella infections of antigenically related and unrelated serotypes, and are in agreement with the work of Smith (1956*b*), Botes (1965), Smith & Halls (1966) and Collins (1968).

Vaccination should be assessed against non-lethal as well as lethal salmonella infections (Collins, Mackaness & Blanden, 1966). Our aim was to show that vaccination reduced the severity of a non-lethal challenge infection in chickens, since they constitute the major reservoir of salmonellas in animals and are suitable laboratory models. The course of an infection was followed by examining groups of chickens at intervals after challenge and measuring the salmonella content of the livers. The infection (i.e. the growth of the pathogen in the liver) was less severe in vaccinated chickens than in controls.

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MATERIALS AND METHODS

Mice

C. F. W. mice bred on the premises were fed on a pellet diet (E. Dixon and Sons Ltd., Ware, Herts.) and weighed 18–20 g. when vaccinated. Food and water were available *ad lib.*

Chickens

Day-old hybrid cockerel chicks, cross Rhode Island Red and Light Sussex (Scientific Animal Services, Elstree, Herts.) were housed in a brooder and at 14 days of age were transferred to wire-floor cages in batches of ten. They were fed on baby chick mash (British Oil and Cake Mills Ltd., Erith, Kent) containing no antibiotics. Food and water were available *ad. lib.*

Vaccine

Freeze-dried *S. dublin* A.T.C.C. No. 15480 (Strain 51, Smith, 1965) vaccine was reconstituted in sterile water when required and 0·2 ml. administered subcutaneously to mice and chickens. Alternatively 1·0 ml. of vaccine in an antacid suspension (Smith, 1965) was given orally to chickens or an appropriate dilution of the vaccine made so that each chicken would take approximately 5 ml. when the vaccine was administered in the drinking water. The vaccine doses are recorded with each experiment. Sensitivity of the vaccine strain to antibiotics was checked against Multodisk 30–9B (Oxoid Ltd., London, S.E.1).

Challenge routes

Oral. Animals were kept without food for 8 hr. and given by mouth an aqueous suspension of the challenge strain with an antacid powder (Smith, 1965). The challenge suspension was contained in 0·2 ml. for mice and 1·0 ml. for chickens.

Intraperitoneal. The same procedure was adopted as for the oral challenge but no antacid powder was added and the challenge dose was contained in 0·2 ml. for mice and chickens.

Challenge organisms

The challenge organisms, known to be pathogenic to mice, were chosen from four groups containing different antigens. They were Group B, *S. typhimurium* 1, 4, 5, 12:i:1, 2, (isolated from a human source in Hammersmith Hospital), Group C, *S. choleraesuis* 6, 7:c:1, 5. (N.C.T.C. No. 5735) and strain S418/68 (Reading) var *kunzendorf* (supplied by Dr W. J. Sojka of the Central Veterinary Laboratory, Weybridge, Surrey). Group D, *S. dublin* 1, 9, 12:gp:(N.C.T.C. No. 9676) and Group E, *S. anatum* 3, 10:eh:1,6. (N.C.T.C. No. F7078/66). These strains were grown overnight on nutrient agar and the challenge suspensions standardized on an E.E.L. colorimeter against a standard curve. The challenge dose was varied according to the particular strain of salmonella so as to kill all the non-vaccinated control mice in 5–6 days. Chickens were challenged with *S. typhimurium* var *copenhagen* (phage type No. 14) isolated from a poultry pro-

cessing plant. It was grown in broth at 37° C. on a swirler for a few hours or overnight and the appropriate dilution of the suspension administered. Viable counts were carried out to determine the number of organisms per dose.

Bacterial examination of tissue for salmonellas

Tissue was examined shortly after death or within a few hours. Either the whole liver of mice and young chicks or a 10 g. sample was examined. The sample was weighed, sterilized by dipping in 0·2 % hypochlorite for 20 sec. and washed in sterile 0·2 % sodium thiosulphate for a further 20 sec. Sterile techniques were employed subsequently. Tissue was sliced and then macerated to liberate bacteria at 14,000 rev./min. for 4 min. in 20 ml. 0·1 % yeast extract in the 100 ml. vessel of the M.S.E. Homogeniser (Measuring and Scientific Equipment Ltd., Crawley, Sussex). Test suspensions of *S. dublin* survived this treatment without any loss of viability. Salmonellas are reported to survive better in diluents containing 0·1 % yeast extract or peptone than in distilled water or saline.

Direct plating of the homogenized tissue was used to measure the number of live salmonellas per gram of tissue. Serial tenfold dilutions were made in 0·1 % yeast extract to 10^{-4} or even higher dilutions, depending on the degree of infection expected. Suitable small volumes were spread on the surface of well dried plates of a selective medium which were incubated overnight at 37° C. If only very small numbers of organisms were expected, duplicate or quadruplicate plates were spread with undiluted suspension in order to examine an adequate amount of tissue. The remainder of the tissue was added to 50 ml. enrichment medium. Direct plating generally revealed salmonellas in all the infected samples. Some samples were positive after enrichment where the infection was minimal and no salmonellas were detected on direct plates. Liver samples often revealed almost pure cultures of salmonellas so that identification presented no difficulty.

Media

Agar plates

The medium contained (per l.) 10 g. yeast extract (Oxoid L21); 2·5 g. bile salts (Oxoid L55); 2 g. tripotassium citrate monohydrate; 5 g. sodium thiosulphate pentahydrate; 15 g. agar (Oxoid No. 1 L11); 2·5 g. lactose; 2·5 g. sucrose; 20 mg. neutral red; 0·2 g. ferric citrate; alkali to pH 7·0 and distilled water to 1 l. The medium was autoclaved at 10 lb. pressure for 10 min. Lactose, sucrose and neutral red were sterilized separately by filtration through a Millipore filter and ferric citrate was autoclaved separately. These constituents were added to the molten agar just before the plates were poured.

Enrichment medium

The selenite mannitol broth contained (per l.) 5 g. peptone (Evans); 4 g. mannitol; 4 g. sodium biselenite; 12 g. dipotassium hydrogen phosphate, acid to pH 6·8 and distilled water to 1 l. The medium was dispensed in 10 or 50 ml. amounts, and steamed for 5 min.

The enrichments were incubated overnight at 43° C. and plated on the selective agar medium. A further enrichment was made by inoculating 0·04 ml. into 10 ml. selenite, and was usually plated after 8 hr. incubation.

Field trials

Examination of poultry houses for salmonellas

After intercrop disinfection and cleansing, twelve dust samples were examined from each house, three each from the floor, walls, beams and ventilators. As each house was restocked, swabs were taken from the chick boxes. Samples of the food and some chicks were also taken to the laboratory for examination. Enrichments were made of liver and caeca for salmonellas and aliquots of the homogenized liver were plated.

Vaccination

Freeze-dried vaccine was added to the drinking water, allowing one font containing 2–3 pints of water to 200 chicks. This quantity was drunk within 3–4 hr. and it is estimated that each chick received 10^7–10^8 cells of the vaccine strain.

RESULTS

Mouse protection tests

Four groups of 10 mice were vaccinated orally or subcutaneously with a single dose of vaccine. Each group was challenged intraperitoneally 14 days later together with non-vaccinated controls with a pathogenic strain of salmonella and the deaths recorded daily for 10 days (Table 1).

Table 1. *Survival of mice against lethal salmonella infections*

Challenge dose (10^6)	Vaccination (oral or subcutaneous)	Vaccine dose (10^6)	Survivors out of 10 at 10 days: Vaccinated	Survivors out of 10 at 10 days: Non-vaccinated	Day on which all non-vaccinated mice were dead
S. choleraesuis	Oral	40	6	0	6
(100 cells)	s.c.	5	6	0	6
S. dublin	Oral	50	6	0	5
(50 cells)	s.c.	25	8	0	5
S. typhimurium	Oral	50	5	0	5
(1·25 cells)	s.c.	25	9	0	5
S. anatum	Oral	50	4	0	6
(250 cells)	s.c.	25	7	0	6

The results show the number of mice surviving at 10 days after challenge. In most cases, half or more than half of the vaccinated mice survived a heavy challenge infection which had killed all the controls in 5 or 6 days. Oral vaccination was almost as effective as subcutaneous vaccination. There was no significant difference in response of the vaccinated mice to challenge by serotypes which were related or unrelated antigenically to the vaccine strain.

Table 2. *Progress of an experimental* S. choleraesuis *var* kunzendorf *infection in livers of mice*

Days after challenge	Log_{10} No. of salmonellas/g. of liver										Deaths	
	Vaccinated					Non-vaccinated					Vaccinated	Non-vaccinated
2	0	0	+	1·14	2·34	0	+	+	2·70	4·22	1	0
3	—	—	—	—	—	—	—	—	—	—	1	0
4	—	—	—	—	—	—	—	—	—	—	2	6
5	—	—	—	—	—	—	—	—	—	—	0	10
6	0	3·16	3·85	4·06	4·73	5·29	5·41	6·77	6·86	> 7	0	6
7	—	—	—	—	—	—	—	—	—	—	1	5
8	2·38	2·29	2·33	2·59	5·32	4·37	6·47	8·06	8·26	9·0	0	0
9	—	—	—	—	—	—	—	—	—	—	0	7
15	2·21	2·77	> 3	3·45	> 5	4·91	None left				1	—
18	0	+	3·27	3·42	5·61	.	.	.	.	.	2	—
25	2·41	2·42	3·30	4·12	5·96	.	.	.	.	.	2	—
32	0	0	2·58	5·89	—	.	.	.	.	.	0	—

Progress of an experimental S. choleraesuis *var* kunzendorf *infection in livers of mice*

Forty-four mice were vaccinated subcutaneously with a single dose of 10^7 cells of vaccine. Fourteen days later, these and 50 non-vaccinated mice were challenged intraperitoneally with about 100 cells from an overnight nutrient broth culture of *S. choleraesuis* var *kunzendorf* after passage through a pig.

At 2, 6, 8 and 15 days after challenge, five mice from each group were killed for measurement of the salmonella content of the liver (on day 15 there was only one left for this purpose in the control group). In the vaccinated group five mice were also killed on days 18 and 25, and the last remaining four on day 32. The salmonella counts in the livers, together with the number of mice that died as a result of the challenge dose are shown in Table 2. There were more deaths amongst the non-vaccinated controls than in the vaccinated mice.

We have assessed the degree of infection in the surviving mice by measuring the salmonella content of the liver by direct plate count. The mesenteric lymph nodes might contain the highest concentration of salmonellas but a quantitative estimation would be impracticable. The figures show a 400-fold difference in the salmonella content of the livers from the two groups.

Salmonella infections in unvaccinated chickens

Lethal challenge infections are often used to test the efficacy of vaccination and the presentation of the results is simple (death or survival). A natural salmonella infection is not necessarily lethal and we have compared the number of salmonellas per gram of a selected tissue (the liver) in vaccinated and non-vaccinated chickens. The liver was selected because salmonellas frequently collect in this organ.

Chickens were challenged intraperitoneally at four weeks of age with graded doses of *S. typhimurium* and two weeks later they were killed and the livers examined. The number of *S. typhimurium* cells found per gram of liver ranged

Table 3. *The number of* S. typhimurium *found in liver after intraperitoneal challenge*

(Five chickens were given the same challenge, and the livers were examined 2 weeks later, and the results are expressed as the number of *S. typhimurium* cells/g. wet weight of liver.)

No. of *S. typhimurium cells* in challenge dose				
40	200	1,000	5,000	25,000
0	40	16	10	125
+	50	18	70	205
750	1,000	405	1,040	375
2,870	3,000	6,350	2,000	900
$> 10^4$	$> 10^4$	u/c	7,150	1,060

\+ No salmonellas were isolated on direct plating but were isolated after enrichment.
0 No salmonellas were isolated after direct plating or enrichment.
u/c The plates were overcrowded and the colonies uncountable.

from none to several millions and bore no relation to the size of the original challenge dose (Table 3) which had spanned a 600-fold range. Five chickens receiving the same challenge dose had such widely different numbers of salmonellas that an average figure would have been meaningless.

Other chickens, varying in age from one to twelve days, were challenged orally with *S. typhimurium*. Each group of four birds received a 1000-fold range of challenge dose. The birds were killed eleven to fourteen days after challenge and the livers examined. The number of salmonellas per gram of liver was independent of the size of the challenge dose (Table 4) and varied widely from one bird to another.

Table 4. *The number of* S. typhimurium *found in liver after oral challenge*

Four chicks received a 1000-fold range of challenge. The livers were examined subsequently and the results are expressed as the number of *S. typhimurium* cells/g. wet weight of liver.

Age of chick at challenge (days)	Age of chick at examination (days)	*S. typhimurium** challenge dose (10^6 cells)	Range of challenge dose			
			× 1	× 1/10	× 1/100	× 1/1000
1	15	0·7	25,000	1,116	240	1,225
2	16	0·8	345	2,200	65	11,000
4	18	0·5	140	35	0	10,000
8	19	0·5	270	6,650	4,930	1,330
10	22	0·55	345	315	8	310
12	24	0·3	165	1,335	90	1,910

* A viable count was performed on each broth culture and tenfold dilutions were made.

Vaccination

Twenty-five chickens were vaccinated orally and 25 subcutaneously at 2 weeks of age, with a range of doses of *S. dublin* vaccine. Two weeks later these and 25 non-vaccinated controls were challenged with different amounts of *S. typhimurium*. They were examined 3 weeks after challenge and the number of *S. typhimurium* per gram of liver estimated (Table 5). All chickens appeared to be in good health and without any visible signs of infection. The challenge organism was considerably reduced or eliminated from the livers of the vaccinated chickens, whereas most of the non-vaccinated were heavily infected. Within the range tested, vaccination was equally effective whether administered orally or subcutaneously and was independent of the size of the vaccine dose.

A more detailed inspection of these results shows the number of salmonellas per gram of liver was much lower in the vaccinated than in the non-vaccinated chickens. The proportion of vaccinated and non-vaccinated chickens falling into various ranges of infection is shown in Table 6.

The figures obtained for the controls (non-vaccinated) (Table 5) are assembled and arranged in order of increasing salmonella content. From this series the proportion of livers whose salmonella content does not exceed a stated value can be plotted as a cumulative frequency diagram or 'ogive' (Fig. 1). The line represents the range of infection amongst the results examined.

The results from the vaccinated chickens are similarly plotted. If vaccination had not been effective, then the two lines would have been superimposed. In Fig. 1 the plot for vaccinated birds is shifted away from the controls down towards the abscissa, a distance of approximately 3 logarithmic units, i.e. a 1000-fold decrease.

Table 5. *The number of* S. typhimurium *cells found in livers of vaccinated and non-vaccinated chickens after a* S. typhimurium *challenge*

(Chickens were vaccinated at 2 weeks of age, challenged 2 weeks later and examined after 3 weeks. Results expressed as the number of *S. typhimurium* cells/g. wet weight of liver.)

Vaccination	Vaccine dose. No. of *S. dublin* cells (10^6)	Challenge dose. No. of *S. typhimurium* cells				
		25,000	5,000	1,000	200	40
Oral	5,000	+	0	4	+	4
	2,500	0	0	0	+	0
	1,250	0	+	–	14	0
	625	+	210	0	8	0
	312	6	+	0	0	+
Subcutaneous	2,000	0	0	30	0	4
	1,000	0	0	0	0	0
	500	0	0	0	20	0
	250	3	0	15	9	0
	125	0	0	10	0	0
Controls	Non-vaccinated	375	1,040	u/c	3,000	0
	Non-vaccinated	205	7,150	405	1,000	+
	Non-vaccinated	900	70	6,350	$> 10^4$	$> 10^4$
	Non-vaccinated	1,060	2,000	16	55	2,870
	Non-vaccinated	125	10	18	40	750

u/c Uncountable, plates overcrowded.
\+ No salmonellas were isolated on direct plating, but were isolated after enrichment.
0 No salmonellas were isolated after direct plating or enrichment.

Table 6. *The proportion of chickens with various ranges of infection*

Range of infection (No. of salmonellas/g. liver)	Proportion of infected chickens (%)	
	Vaccinated	Non-vaccinated
0	58	4
0–10	90	12
0–100	96	32
0–210	100	40
0–1,000	100	60
0–5,000	100	80
0–10,000	100	88

Progress of an experimental infection

Twelve chickens were vaccinated by mouth (5×10^8 cells of the vaccine strain) when 5 days old and 14 days later were challenged intraperitoneally, together with 12 non-vaccinated controls, with 470 *S. typhimurium* cells. The course of the

infection was traced by examining the livers of vaccinated and non-vaccinated chickens at intervals after challenge (Fig. 2). The experimental infection increased rapidly to a high level in the controls and then slowly regressed. The infection was considerably reduced in vaccinated chickens. With one exception, the infection in the vaccinated chickens did not exceed 70 salmonellas per gram, whereas at the peak of infection some non-vaccinated controls had more than 3000 salmonellas

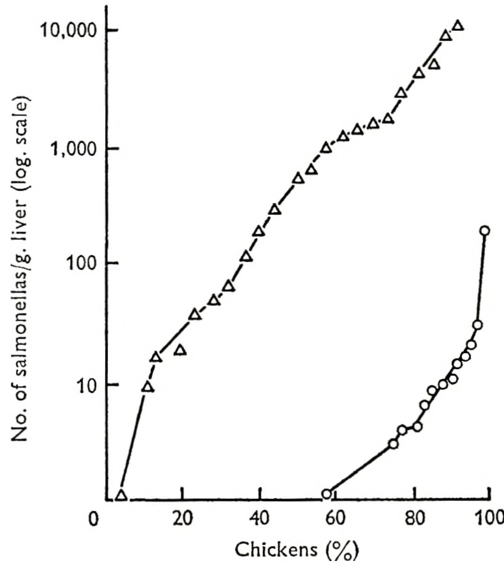

Fig. 1. Effect of vaccination on the salmonella content of livers of infected chickens. The number of salmonellas/g. liver was measured in vaccinated (○) and non-vaccinated (△) chickens.

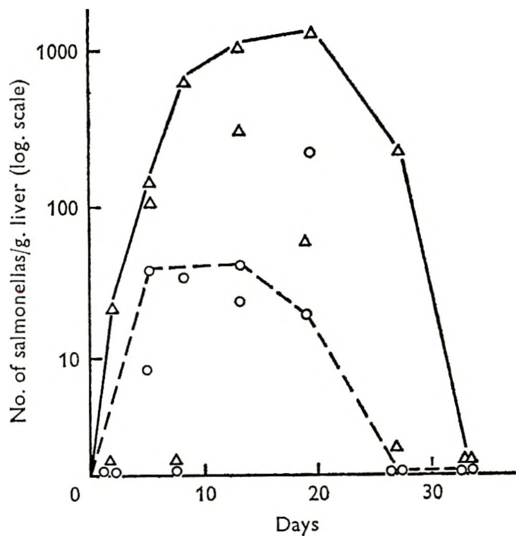

Fig. 2. Progress of a *S. typhimurium* infection in livers of vaccinated (○) and non-vaccinated (△) chickens.

per gram of liver. A comparison between the two groups is shown as an ogive in Fig. 3. Again vaccination had displaced the vaccinated curve downwards more than 1 logarithmic unit. No salmonellas were found in 5/12 vaccinated chickens compared with 1/12 of the controls. Of the vaccinated chickens, 92 % had no more than 100 salmonellas per gram of liver compared with 25 % of the controls. Half the vaccinated chickens had fewer than 10 salmonellas per gram, whereas half the controls had more than 400 per gram. One quarter of the controls had more than 1000 salmonellas per gram of liver.

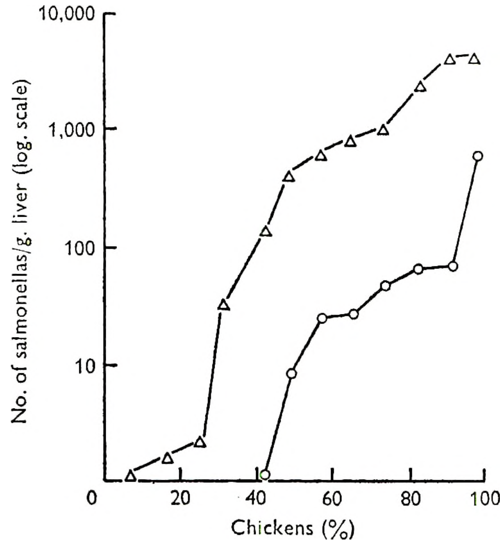

Fig. 3. Effect of oral vaccination on the salmonella content of livers of *S. typhimurium* infected. The number of salmonellas/g. liver was measured in vaccinated (○) and non-vaccinated (△) chickens.

A comparison of oral and subcutaneous vaccination

Chickens 3 days old were vaccinated orally with 1×10^8 cells of *S. dublin* vaccine in antacid suspension, and chickens 9 days of age were vaccinated with 1×10^7 by subcutaneous injection. Together with control non-vaccinated chickens they were challenged at 18 days of age with *S. typhimurium* either 250 organisms intraperitoneally or 1×10^6 orally. Four chickens from each group were examined at intervals until the infection could not be detected in the controls.

The distribution of infection in both groups is shown as a cumulative frequency diagram in Fig. 4. The effect of vaccination was to displace the vaccinated curve as in Figs. 1 and 3 about 2 logarithmic units. Oral and subcutaneous vaccination were equally effective.

Effect of vaccine in drinking water

Since oral and subcutaneous vaccination were equally effective, the vaccine might also be effective if added to the drinking water thus eliminating the need for injection. The vaccine was resuspended in sufficient drinking water so that each

day-old chick would receive 10^8 viable cells by drinking 5 ml. of water over a period of several hours. At the same time other chicks were vaccinated with a similar dose of vaccine *per os*.

Groups of chickens from each method of vaccination together with control non-vaccinated chickens were challenged orally with *S. typhimurium*. This experiment gave results similar to those in Fig. 4 and showed that oral and drinking water vaccination were equally effective.

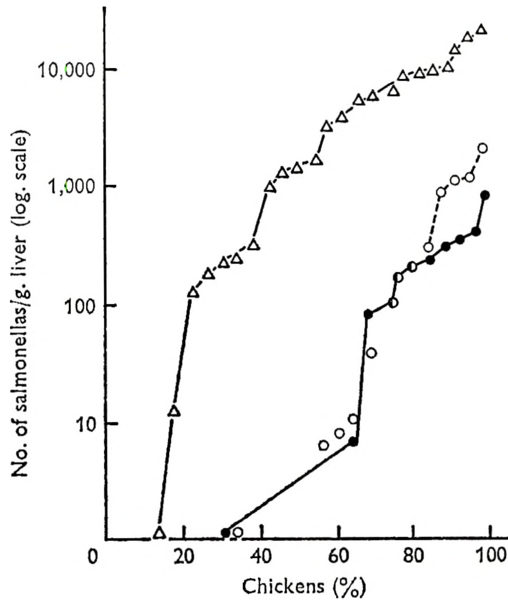

Fig. 4. Effect of subcutaneous or oral vaccination on the salmonella content of *S. typhimurium* infected chickens. The number of salmonellas/g. liver was measured in non-vaccinated (△), orally vaccinated (○) and subcutaneously vaccinated (●) chickens.

Some properties of the vaccine in chickens

Use of the vaccine in the field

Litter and dust were examined from several farms and two of these from which *S. typhimurium* was isolated were chosen for trials. Newly hatched chicks received the vaccine in the drinking water on arrival. Some tagged control chicks were segregated and did not receive any vaccine, but were allowed to mingle with the vaccinated birds 10–14 days later. Subsequently some livers from each group were examined for the presence of *S. typhimurium*.

Farm No. 1. Although five out of six broiler houses on this farm had previously been infected, no evidence for infection appeared during the trial. *S. typhimurium* was isolated from only one out of 95 chicks examined, but was not isolated from dust, litter, food or swabs taken from the newly hatched chicks. The thorough cleansing and disinfection had removed the infection.

Farm No. 2. One hundred chicks from the main flock were brought back to the laboratory and half of them were vaccinated and half left as controls. Two weeks

later they were all challenged with 10^6 *S. typhimurium* cells and subsequently examined. In the laboratory trial, the challenge organism was detected only after enrichment in three out of fifty livers from vaccinated chickens whereas it was present on the direct plates of twelve of the controls. On the farm, however, *S. typhimurium* was detected in only one out of one hundred chickens examined.

Tolerance

Twenty-five 1-day-old chicks tolerated 50 times the recommended vaccine dose and no deaths were recorded. More than 160,000 day-old chicks have been vaccinated on farms and in laboratory tests and no ill effects have been observed.

Persistence of vaccine strain

To determine how rapidly the vaccine strain was destroyed, chickens were orally vaccinated with one or five times the recommended vaccine dose (10^8 cells). Cloacal swabs or livers were examined at intervals up to 40 days. The vaccine strain was isolated from 55 % of chickens examined from 1 to 4 days, in 25 % from 5 to 8 days and less than 1 % from 9 to 40 days.

Effect of passaging the vaccine strain

Chickens were vaccinated and the organism re-isolated and 5×10^8 cells injected subcutaneously into fresh chickens. After four such passages the organism remained unchanged and no ill effects were noted in any of the chickens.

Viability of the vaccine strain in the drinking water

The viability of the vaccine strain was measured 4 hr. after the freeze-dried organisms were re-constituted in distilled water or London tap water supplemented with varying amounts (0–0·2 %) of sodium metaphosphate (Calgon). The viability of the vaccine strain fell rapidly in London tap water and no survivors were found after 4 hr in comparison with distilled water where 80 % of the cells remained viable. The addition of Calgon (0·05; 0·1 and 0·2 %) to London tap water produced 24, 64 and 76 % of the initial viable count. Owing to the variability of water supplies and the presence of bactericidal agents, it is recommended that 0·2 % Calgon be added routinely to avoid undue loss of viability of the vaccine strain.

Antibiotic sensitivity

The vaccine strain was resistant to erythromycin, novobiocin, cloxacillin, penicillin, streptomycin, tetracycline and sulphafurazole, but it was sensitive to chloramphenicol, ampicillin, neomycin, kanamycin and furazolidone. The effect of furazolidone in the diet of vaccinated birds was studied. The results suggested that furazolidone should not be introduced earlier than 4 days after vaccination. The introduction of furazolidone later than 4 days after vaccination appeared to be beneficial and these birds were less infected than the controls not receiving the drug.

DISCUSSION

The effect of vaccination was to reduce the salmonella content of chicken livers significantly. Reductions from tenfold to a 1000-fold were observed. The liver was chosen for quantitative examination for three reasons; it provided a convenient way of assessing the number of salmonellas per gram; it contained the highest concentration of salmonellas, and infected chicken livers may cause outbreaks of food poisoning. This work has shown that vaccination could reduce this hazard.

Until recently, live vaccines were not favoured because of the fear that they might become virulent. With the use of the *S. dublin* vaccine in calves, Hall & Taylor (1970) found little evidence of the strain appearing in human or animal sources and concluded that there was no danger to the general public.

Chickens are most susceptible to invasion and infection by salmonellas during the first few days of life and become more resistant to infection as they grow older. Since vaccination with a live oral vaccine is equivalent to a mild infection, it should be given as early as possible after hatching in order to anticipate a natural challenge. Incorporating the vaccine in the drinking water avoids the need for injections.

Because salmonellosis in flocks is sporadic we did not encounter a field challenge whilst testing the vaccine on farms. However the laboratory trial which was carried out in parallel with the field trial on farm 2 confirmed our earlier findings and further work on these lines with Houghton Poultry Research Station will be published elsewhere.

We wish to thank Miss J. A. Bishopp, Mr K. R. Comber and Mr J. S. Thurgood for their valuable technical assistance in this work.

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Lizards as vectors of human salmonellosis

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SUMMARY

Human infections with *Salmonella saintpaul* have become more frequent in New Zealand in recent years. Most cases now occur in Otago. It is shown that wild lizards in Otago, particularly the common skink (*Leiolopisma zelandica*), carry *S. saintpaul* and that most if not all human outbreaks of this salmonella serotype are associated with either lizards or lizard infested areas. So far as is known this is the first report incriminating lizards as the probable cause of human salmonellosis.

INTRODUCTION

Salmonella saintpaul infection in humans was first confirmed in New Zealand in 1952. Occasional sporadic cases were observed throughout both North and South Islands since that date, but from 1959 onwards a large proportion of the cases have been in the South Island and most of them have been associated with Otago. Of the 34 human index cases recorded in New Zealand, 18 have occurred in Otago. Since 1959 69 % of recorded human index cases have been associated with Otago and since 1966 the proportion has risen to 81 %. The age range has been wide but the infection has been mainly in children, particularly from 6 months to 10 years of age. Most Otago cases have occurred in the summer season from November to April, though one infection each has been recorded for July, September and October.

In 1966, after the notification of five apparently unconnected cases of *S. saintpaul* infection in Otago an intensive investigation was carried out in an endeavour to trace a common origin. Four of these cases were associated with an area of the Clutha river running through Central Otago from Alexandra to Roxburgh. Sanitary surveys were undertaken and many water, soil, domestic animal and even vegetable samples were collected in a fruitless endeavour to find the source of these cases.

In November 1967 a 3-year-old boy was admitted to Kew Hospital, Invercargill, severely ill with *S. saintpaul* infection. Examination of the faeces of every member of the household as well as two domestic helpers showed tham all to be carriers. Eighteen other human contacts and a wide variety of domestic animals and birds

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were negative on stool culture. However, a biscuit-tin, kept in the kitchen, housed five skinks (*Leiolopisma zelandica*). The excreta of these lizards yielded an almost pure culture of *S. saintpaul*. These five lizards were the survivors of some 48 lizards which had been collected the previous month near Clyde, not far from Alexandra.

Following this discovery a collection was made in December 1967 of 30 skinks (*L. zelandica*) and 20 geckos (*Hoplodactylus pacificus*) from the same valley where the original 48 lizards had been found. Three of the wild skinks were found to be excreters of *S. saintpaul*.

A review now took place of all previous notifications of human *S. saintpaul* infections occurring in Otago. It was then established that a history of association with the handling of wild lizards or with earth, rocks or vegetation in areas where lizards abounded, was present just before the onset of the human illness in every case except one in which the source of infection had been established as arising from a goose which the patient had eaten. This goose, however, was traceable to a farm in Macraes Flat, North Otago, and was known to have eaten lizards before it was killed for the table.

A review of the human *S. saintpaul* infections (supplied by the National Health Institute) occurring in other Districts of New Zealand was not so profitable. Many of the cases had either not been notified to the local Medical Officer of Health or the inquiries undertaken at the time were insufficient to establish whether an association with lizards or lizard-infested areas had been present or not before infection. In two cases, however, there was sufficient information to make it highly probable that there had been association with lizards or lizard droppings.

Table 1 summarizes the index cases of the sporadic Otago outbreaks, showing the probable area of original infection.

LIZARD INVESTIGATION

At the end of 1967 and during the early part of 1968, 120 lizards (95 *L. zelandica* and 25 *H. pacificus*) caught wild in Otago and Southland were examined bacteriologically at Kew Hospital, Invercargill. To some extent these early examinations were experimental because it was not known exactly how specimens should be taken nor from what part of the gut. However, it was soon established that it was necessary to kill the animal and take cultures from the upper gut. None of the lizards from Southland yielded *S. saintpaul*, but three of 30 *L. zelandica* from Clyde were positive for this organism. Apart from four specimens collected from Otago Peninsula and kept as pets at a primary school which were examined at Dunedin Hospital, all further animal investigations were undertaken by the Invermay Animal Health Laboratory near Dunedin. Over the summers of 1968–1970 an additional total of 372 lizards (174 *L. zelandica* and 198 *H. pacificus*) were examined at Invermay.

In the early stages, Livestock Instructors of the Department of Agriculture managed to capture lizards for the laboratory but it was soon realized that the job was very time-consuming and by no means easy, interfering considerably with

other more urgent work. It was therefore arranged that Inspectors of Health of the District Health Office would undertake, after prior arrangement with the laboratory, a series of 'forays' for lizard-collecting. The Inspectors worked in pairs and had instructions to take only single specimens where there were many, from as wide a range over the water catchment areas as possible. The large territorial area involved, the rugged upland terrain inhabited by the lizards and the difficult road access to many of the places visited meant that each 'foray' lasted 3 or 4 days.

Table 1. *Outbreaks of* Salmonella saintpaul *infection in humans in the southern half of South Island*

Age of index case	Date	Residence	Probable area of original infection	River catchment
52 years	Apr. 1959	Dunedin	Macraes Flat	Waikouaiti
1½ years	Sept. 1961	Roxburgh	Roxburgh	Clutha
8 weeks	Nov. 1962	Roxburgh	Roxburgh	Clutha
17 years	Jan. 1965	Palmerston	Palmerston	Shag
6 months	Mar. 1965	Roxburgh	Roxburgh	Clutha
5 years	Mar. 1966	Otematata	Otematata	Waitaki
7 years	Apr. 1966	Roxburgh	Roxburgh	Clutha
1 year	Oct. 1966	Dunedin	Alexandra	Clutha
6 years	Dec. 1966	Roxburgh	Roxburgh	Clutha
19 years	Dec. 1966	Roxburgh	Roxburgh	Clutha
3 years	Nov. 1967	Invercargill	Clyde	Clutha
2 years	Feb. 1970	Dunedin	Clyde	Clutha
2½ years	Mar. 1970	Macraes Flat	Macraes Flat	Waikouaiti
41 years	Apr. 1970	Palmerston	Palmerston	Shag
72 years	Apr. 1970	Karitane	Karitane	Waikouaiti
10 weeks	Apr. 1970	Dunedin	Dansey's Pass	Taieri
11 months	July 1970	Ettrick	Ettrick	Clutha
13 years	Nov. 1970	Roxburgh	Roxburgh	Clutha

An efficient system of catching lizards and dispatching them alive to the Invermay Laboratory had to be developed. Learning the techniques of finding 'runs', observing droppings, upturning boulders as well as the acrobatics needed to secure the skinks, which run fast when uncovered, was left to the Inspectors. Since it was essential to prevent possible cross-contamination each lizard had to be 'packed' separately.

The most satisfactory and successful system developed was to put each specimen in a plastic bag with a little air, seal and label the bag with the time, date and place of collection and pack the bags in an ice-box. The cold environment encouraged an artificial hibernation and the lizards travelled alive and well by public transport for upwards of 200 miles. Fifty or more lizards could easily be packed in an ice-box of internal capacity of about 12,000 $cm.^3$. To be successful lizard-hunts were best undertaken in hot sunny weather.

On arrival at the laboratory the lizards were killed and dissected aseptically. Stomach and gut contents were cultured separately on brilliant green and MacConkey agar plates (B.B.L.*). The entire length of stomach and intestine was then

* B.B.L. = Baltimore Biological Laboratories.

inoculated into selenite-F enrichment broth (B.B.L.) and incubated at 42° C. for 18–20 hr., then cultured on brilliant green agar. All plates were incubated at 37° C. and examined at 18 and 48 hr.

Routine biochemical confirmation was carried out by subculturing suspicious colonies on Triple Sugar Iron agar (B.B.L. and Difco) and Christensen's urea slopes. Serological confirmation was carried out using Burroughs Wellcome somatic antisera for slide agglutination tests to determine the salmonella somatic grouping. Flagellar titrations were at first done using Burroughs Wellcome flagellar antisera, later Difco Spicer Edwards pooled antisera and single factor flagellar sera were used.

RESULTS AND CONCLUSIONS

Table 2 gives the combined results of cultures undertaken by Kew Hospital and Invermay Animal Health Laboratory. Geographically by far the largest river catchments are the Clutha and the Waitaki and hence the much greater proportion of specimens collected from these two catchment areas. Among the smallest are the Opihi, the Waihao, the Shag, the Waikouaiti, the Waikawa and the Waituna.

Table 2. *Wild lizard collections from Rakaia River southwards, tested for* Salmonella saintpaul

River catchment		*Leiolopisma zealandica*	*Hoplodactylus pacificus*	Total lizards
South Canterbury	Rakaia	0/2	0/14	0/16
	Ashburton	2/17 (12)	2/10 (20)	4/27 (15)
	Rangitata	1/9 (11)	0/9	1/18 (6)
	Opihi	0/2	0/8	0/10
	Waihao	0/2	0/3	0/5
	Sub-total	3/32 (9)	2/44 (5)	5/76 (7)
Otago	Waitaki	3/49 (6)	1/103 (1)	4/152 (2)
	Shag	2/9 (22)	0/0	2/9 (22)
	Waikouaiti	4/16 (25)	0/3	4/19 (21)
	Taieri	1/19 (5)	0/6	1/25 (4)
	Clutha	11/79 (14)	1/62 (2)	12/141 (9)
	Sub-total	21/172 (12)	2/174 (1)	23/346 (7)
Southland	Waikawa	0/10	0/0	0/10
	Mataura	0/25	0/5	0/30
	Waituna	0/25	0/0	0/25
	Oreti	0/5	0/0	0/5
	Sub-total	0/65	0/5	0/70
	Totals	24/269 (9)	4/223 (2)	28/492 (5·7)

Figures indicate no. positive/no. tested, with percentages in parentheses.

Of the Otago river catchments (the Waitaki, the Shag, the Waikouaiti, the Taieri and the Clutha) each reveals an enzootic of *S. saintpaul* infection amongst the skinks and a suggestion of a much lower infection rate amongst the geckos. The broad conclusions were reached that skinks are more frequent carriers of *S. saintpaul* than geckos and that the carrier-rate amongst skinks in Otago appears higher than in the provinces to the north or south. Why this should be is as yet unknown

and clearly a more widespread and extended survey of the *S. saintpaul* carrier state amongst New Zealand lizards should be undertaken.

A possible explanation of the largest numbers of human cases having arisen from Central Otago in recent years is that this area attracts very large numbers of summer holiday-makers, the weather and the terrain being attractive to both humans and lizards. However, the recent cases occurring in the Waikouaiti and Shag River catchments, which are not considered holiday resort areas, throw some doubt on this hypothesis.

It has been suggested that the reason for the apparently high incidence of *S. saintpaul* infection in Otago is due to greater enthusiasm amongst Otago medical practitioners for taking faecal specimens in cases of diarrhoea. However, it seems unbelievable that in other parts of the country faecal cultures are not always taken of children suffering from severe bloody diarrhoea. Otago has a relatively small population but its proportion of all recorded *S. saintpaul* infections in New Zealand is greatly in excess of what would be expected on a population basis. Moreover, it is today very unlikely that any hospital laboratory throughout the country will confuse *S. typhimurium* with *S. saintpaul.*

This paper does not pretend to show conclusive evidence that the lizards themselves are always responsible for all human cases of *S. saintpaul* infection. The evidence, however, is strong that human infection rates are highest where skink carrier rates are highest.

Undoubtedly the actual handling of lizards is not necessary for infection to be obtained. It is apparently sufficient for close contact with earth or rocks in an area abounding with lizards to cause infection. Since it has been established that lizard excreta may contain very large numbers of the organisms it is possible that *S. saintpaul* might be picked up on hands or clothing and thereby cause infection by the oral route. The work of Wilkoff, Westbrook & Dixon (1969) on the ability of *S. typhimurium* to survive for long periods on fabrics would suggest that *S. saintpaul* might also be picked up on clothing, even perhaps from sitting on the ground contaminated with lizard excreta.

DISCUSSION

The evidence presented here, while not conclusive, is sufficient to allow for prima facie belief that lizards of the species *Leiolopisma zelandica* and *Hoplodactylus pacificus* are vectors in human salmonellosis. A search of the literature on salmonellas has failed to reveal any previously recorded cases of human salmonella infection which can be attributed to infection by members of the lizard group.

Isolations of various salmonellas from lizards have been made on a number of occasions. McNeil & Hinshaw (1946) reported *S. manhatten* from a zoo iguana and *S. montevideo* from a Gila Monster. They considered their report to 'be the first true salmonellae to be reported from lizards', although drawing attention to the work by Caldwell & Ryerson (1939) on salmonellosis in reptiles. Mackey (1955) in reporting lizard isolations from 1948 to 1953 in Dar es Salaam isolated 33 different salmonella serotypes. This formidable list included *S. typhimurium* but not

S. saintpaul and the organisms were apparently cultured and identified from droppings of two species of common house lizard, *Hemidactylus mabonia* and *Mabuya striata*. He makes the pertinent observation that 'it is difficult to understand how the lizards become infected'.

Other salmonella isolations from lizards include one serotype from the Pacific fence lizard (*Sceloporus occidentalis*), by Hinhsaw & McNeil (1947); one in African lizards (Van Oye, 1964); and ten different serotypes in Australian lizards by Atkinson (1964). The only references to the isolation of *S. saintpaul* from reptiles were found in articles by Refai & Sadek (1968) where it occurred in *Cerastes cerastes*, a horned viper, and by Iveson, Mackay-Scollay & Bamford (1969) in Western Australia where it was isolated from the two lizard species, *Varanus varius* and *Tiliqua scincoides*.

A number of authors stress the potential importance of lizards as vectors or carriers of salmonellas, e.g. Collard & Montefiore (1956) in *Agama agama* in Ibadan, and Le Minor (1964) who found 19 out of 497 *Hemidactylus bleker* and 14 out of 152 *Peripia peronii* positive for salmonellas. Outbreaks of salmonellosis in captive lizards have been reported by Lee & Mackerras (1955). Darasse, Le Minor & Lecompte (1958) point out the potential danger of contamination of drinking water supplies from lizard salmonellae.

The first isolation of *S. saintpaul* from a human in New Zealand was made in Rotorua in 1952 (Josland, 1953). That author considered it an uncommon infection in both man and animals at that time. Since then reports of *S. saintpaul* epidemics in man, such as that by Gotoff, Boring & Lepper (1966), have shown that human infections with this salmonella may be becoming commoner. The present paper would tend to support the view that human infections with *S. saintpaul* are becoming commoner in this country, particularly in Otago, and, so far as can be ascertained, is the first paper to associate infections in man with lizards as the vectors.

We are indebted to Mr R. Beal, Health Inspector, for his discovery that lizards were the probable vectors of the outbreak of *Salmonella saintpaul* infection in Invercargill; to the Livestock Instructors and Inspectors of Health who caught the wild lizards; to Dr N. G. Prentice, Pathologist, and Mr D. B. Pomeroy, Bacteriologist of Kew Hospital, Invercargill who took the brunt of the original bacteriology; to Associate Professor N. Markham and his colleagues in the Department of Microbiology, Dunedin Hospital, and to Dr D. Perry and Dr N. Fitzgerald, Pathologists, for their work on the human cases; Dr J. D. Manning, lately Director of the National Health Institute; and to the Directors-General of Health and Agriculture for permission to publish.

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Common colds on Tristan da Cunha

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SUMMARY

Eight epidemics of respiratory disease have been observed among islanders of Tristan da Cunha. They seem to be initiated by the arrival of ships and transmission seemed to occur as a result of close human contact but could not always be traced. Islanders suffered from less colds than those in less isolated communities.

INTRODUCTION

For many years it has been known that there were some unusual features of the epidemiology of common colds on the island of Tristan da Cunha in the South Atlantic. Epidemics were said to occur which affected many of the islanders, but they only took place when ships called at the island from Cape Town and not from more distant parts (Woolley, 1963). Similar epidemics have been noticed on Easter Island (Heyerdahl, 1958). However, systematic prospective studies had never been made in such a situation.

Tristan da Cunha was evacuated in 1961 because of the eruption of the volcano and the islanders came to Britain, where, after an initial period of unusually frequent and unpleasant respiratory tract infections, the frequency and clinical pattern of illness seemed to be much like that of other inhabitants (Black, Thacker & Lewis, 1963; Samuels, 1963). It was therefore of interest to find out whether the pattern of epidemics observed before the evacuation would be re-established when the islanders returned to Tristan da Cunha and also to document, as far as possible, the occurrence of such epidemics which previously had been based only on impressions and hearsay evidence. For 5 years after the main party returned continuous records of respiratory infections were kept, apart from one short break, and this paper presents a qualitative analysis of these.

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THE POPULATION AND MIGRATIONS

The evacuation and repopulation are shown graphically in Fig. 1. The island was re-occupied as follows:

1962: Scientists of a Royal Society expedition took an advance party of 12 men. When the volcanic activity subsided, 12 islanders returned to the island in January, in company with scientists of a Royal Society expedition.

1963: In May, 52 islanders returned, followed by 198 in November; this left only 14 islanders in Britain.

1966: In April, there was a voluntary migration from Tristan by 37 islanders, who came back to Britain.

1967: In October, 9 of this group decided to return once again to Tristan.

1968: In August, they were followed by another 6.

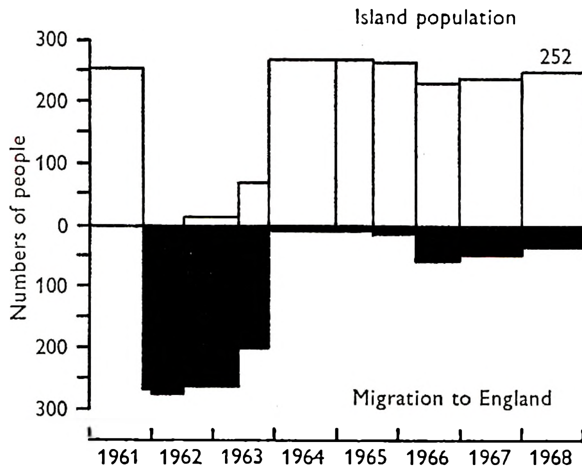

Fig. 1. The movement of islanders to England following the volcanic activity of October 1961, and the relatively stable population of the island following the return of the main party in 1964.

During the period of this study the traditional pattern of life on the island had been re-established and was maintained. Islanders lived in 71 houses, grouped as shown in Fig. 2. The number in each house varied from 1 to 8, the modal number being 3. Their children between the ages of 5 and 15 years attended the school, in which they were divided into 4 classes by age. There is a canteen which the adults visited almost every evening. The men worked outside much of the day but women met together indoors in fairly well-defined 'carding parties' for work and social intercourse. There were usually 40–50 islanders at the church service on Sunday and there were occasional parties at which virtually the whole population gathered under one roof. Apart from this there were a great many casual contacts between neighbours, both indoors and outdoors, though these tended to be more frequent among those who were closely related. Hence, the internal contacts did not differ much from those of well-knit village communities in all parts of the world; however, contacts with the outside world were infrequent. As shown in Fig. 3, ships

called only occasionally. The most important of such visitors were the fishing vessels *Gillian Gaggins* and *Tristania*. These worked in Tristan waters twice a year, namely between September and December and between January and March, when about a dozen islanders joined the crew. The ships took their catches to Cape Town where they spent about a month, the voyage taking about 6 days. These ships, as a courtesy, took occasional passengers to or from the island.

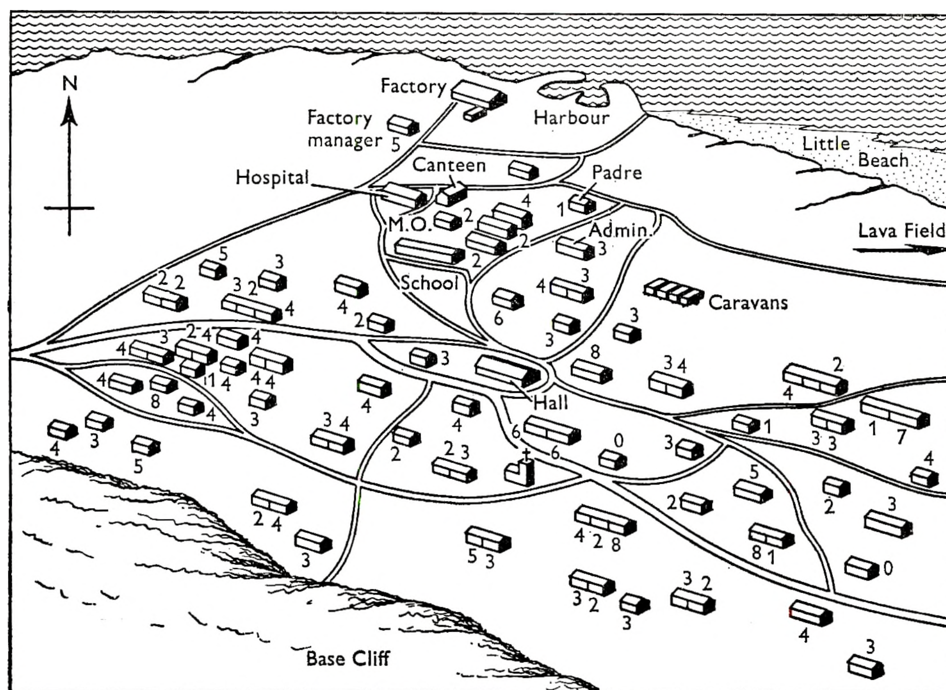

Fig. 2. The housing of the community in 1968. The layout of the village is shown and the digit alongside each building shows the number of people living in each house or part of a house.

In addition to the islanders there were a few expatriate employees on the island. These were the administrator, padre, radio operator, public works department operator, school teacher, doctor and factory manager, and each was accompanied by his family. All had frequent contact with the islanders.

Collection of information

The island medical officers were in continuous residence, S.G. from May 1963 to May 1965 and M.S. from May 1965 to June 1968. They kept ordinary clinical records of patients who consulted them and, in addition, they used the opportunity of their frequent contacts with the islanders to detect as far as possible all members of the community who developed minor upper respiratory infections. Each patient, as recognized, was given a simple diary record card, based on that of Hope-Simpson (see Tyrrell, 1965), which he completed for the period of the illness; other members of the patient's family were given cards also, and completed them if they

became ill. It was not possible to make a complete check of the population, but most of them were thoroughly cooperative and completed cards, although they needed a little assistance at times. It is likely, however, that there was some under-reporting, which may, of course, have varied in amount, being less when the type of illness was more severe and therefore more likely to require medical attention. Apart from this weakness the records seem to be quite adequate for our purposes.

Shipping movements were supplied from the records of the offices of the companies and the administrator of the island.

DESCRIPTION OF THE EPIDEMICS

The epidemics have been summarized in Fig. 3 which shows the general pattern of discrete outbreaks which were reported by previous visitors to the island. Two epidemics were incompletely recorded and are so indicated. The outbreaks tended to occur after the arrival of ships, as shown in Fig. 3, and usually followed the arrival of ships coming directly from Cape Town, rather than those which had been

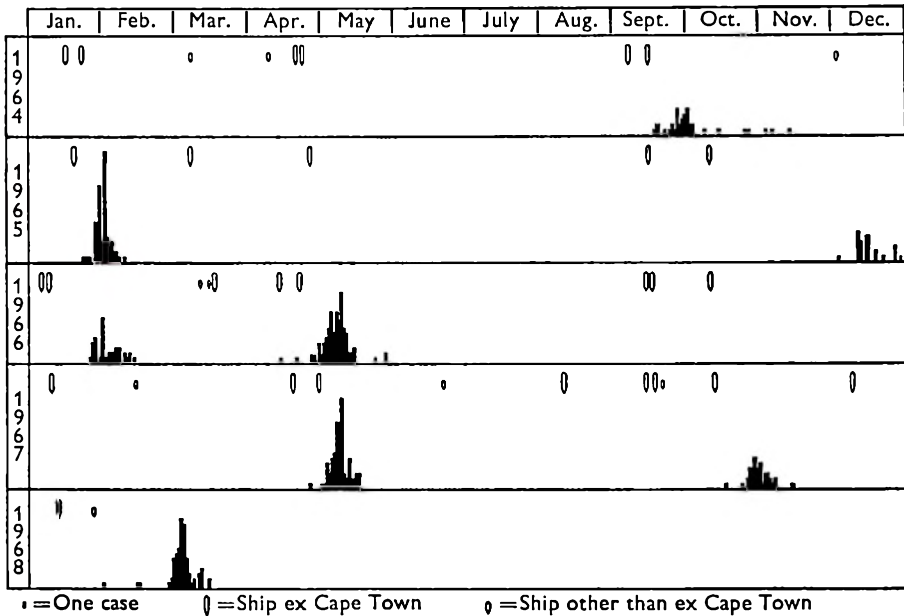

Fig. 3. The occurrence of epidemics of respiratory disease. The arrival of ships from Cape Town is also shown. Almost all colds occurred in definite epidemic waves. Between these waves there were a few sporadic cases, although some of these seemed to be 'leading up' to an epidemic. Epidemics in 1964 and December 1966 were incompletely recorded.

at sea for long periods. We would like to have known whether there were any characteristic circumstances which led to the initiation of an epidemic, but were hampered by lack of information about the occurrence of colds on the ships and the exact details of the coming and going of members of ships' companies and the islanders.

On some occasions the circumstances were obvious; for example, in April 1967 B.L. was returning from Cape Town and developed a cold 2 days before coming ashore. When he got home he passed it on to his family, whence it spread to the community. On the other hand, in October 1967 D.R., a young woman who was not known to visit the ship and who worked in the hospital, was apparently the first case after the arrival of the ship 3 days before. No further case was detected until several days later, when another young woman who probably had no close or direct contact with her developed a cold. Another gap occurred and then an epidemic developed quite rapidly (see Fig. 3).

Relationship between epidemics on board ships and on Tristan da Cunha

Three ships called regularly at Tristan and each made trips twice yearly. Two were small boats which fish for rock lobsters in shallow Tristan waters. These ships brought limited food and emergency supplies for the island, which were off-loaded before fishing. The MV. *RSA* brought the main supplies for the island store and passengers arriving about the end of April and the middle of October from Cape Town. The *RSA* stayed in Tristan waters for from 1 to 2 weeks, but might have had to wait longer if the sea was rough. The passengers who arrive from Cape Town on the *RSA* are often the source of common cold infections on the island. In May 1965, however, the *RSA* called at Gough Island first and did not arrive at Tristan until 3½ weeks later and, on that occasion, no common cold epidemic occurred. It is possible that any virus on board had died out during the 3–4 weeks' journey. The fishing ships, on the other hand, made a more intimate contact with the islanders, in the sense that some islanders were employed to work on board the ships throughout a fishing season.

Table 1. *Clinical features of successive epidemics*

	Jan–Feb. 1966	April–May 1966	April–May 1967	Oct–Nov 1967	Feb–March 1968	All epidemics
Total number	41	104	96	41	68	350
Percentage of total with:						
Nose blocked	88	64	42	49	37	—
Nose running	97	75	75	85	88	—
Throat sore	83	52	45	44	53	—
Cough	85	58	35	56	63	—
Tight chest	51	23	17	24	22	—
Feeling tired	66	28	14	12	12	—
Headache	71	47	19	32	22	—
Median duration (days)	6	6	4	4	5	—

The first fishing season each year started in the middle of January and continued until the middle of March. The second season started in September and finished in the middle of December. Before starting any fishing, about 20 dinghies were collected from the settlement. The two ships usually arrived almost together, but if the sea was rough they waited in the lee of the island for a couple of days. The fishing boats usually worked round Nightingale and Inaccessible Islands which

are small and uninhabited and situated about 40 km. S.W. of Tristan: they also made a fishing trip to Gough Island, 350 km. S.S.E., but they called at the settlement 3 to 4 times for supplies of fresh water and on these occasions the islanders on board visited their families.

These mid-season visits may have been very important in spreading common colds. The islanders worked on board the ships in intimate contact with the Cape Town crew and if there was a common cold epidemic on board the ship, the islanders usually suffered from the infection. For example, in 1965 the *Gillian Gaggins* was newly commissioned and made her maiden voyage to Tristan. About 20 islanders were employed on that occasion to help with fishing, but after a week the refrigerator broke down and she had to go to Cape Town for repairs. Almost half the islanders who came ashore had colds and this initiated the late 1965 epidemic. However, on another occasion, one of the ships did not call at the settlement during the season and instead collected water supplies from Gough Island. Towards the end of the season when the islanders came home finally they admitted to having suffered from common colds in the first 2 weeks of fishing, and this infection was passed on to the members of the meteorological team stationed at Gough. Nevertheless, when the ship called at the settlement about 8 weeks after leaving no epidemic resulted.

In addition to the *Gillian Gaggins*, the *Tristania* and the *RSA*, other ships call at the island from time to time. These include ships of the Royal Navy, passing tourist liners, Norwegian whaling ships and sometimes oil tankers. These can only make contact with the island when the sea is calm and boats can enter the harbour safely. Occasionally, a ship with a serious medical emergency on board may call at the settlement if it is considered risky to head for the destination or the nearest mainland port. Cold epidemics are practically never initiated by such contacts.

Comparison of the epidemics

The only epidemics which occurred on the island were the epidemics of respiratory diseases shown in Fig. 3. Nevertheless, these varied from each other in a number of ways. A summary of the symptoms reported in five epidemics is given as Table 1. The number of cases which we recorded ranged from 41 to 104 – from about one-fifth to one-half of the islanders. The epidemics also varied in duration, some being over quickly, while others lingered on (Fig. 3). The first epidemic, though small, was associated with much sore throat, cough, headache and malaise as well as coryza. In all epidemics, nasal discharge was the most frequent symptom, followed by cough, sore throat and nasal obstruction. In the 1966 epidemics, the illness lasted longer as well as being accompanied by more constitutional upset. In the two epidemics that were incompletely recorded, sore throat was a prominent symptom.

During epidemics, a number of patients with mild asthma or chronic bronchitis attended the hospital because their symptoms had worsened and a number of these had recently suffered from common colds.

It can, therefore, be concluded that the eight epidemics observed between 1963 and 1968 were fairly uniform in clinical pattern, varying a little in severity and in

the prevalence of sore throat. Since less than half the population was involved in any one epidemic, it is clear that on the average the islanders were suffering from less than one cold a year, substantially less than the inhabitants of less isolated rural or urban communities.

The records of each illness showed the presence or absence of each symptom on each day. It was therefore possible to arrange the information so as to show how many subjects on each day were showing symptoms of a cold. These figures are being used in studies by B. E. Hammond (in preparation).

DISCUSSION

We feel that the study of this isolated community has been of value in obtaining fairly clear evidence that acute respiratory disease in a very isolated small community behaves as an infectious disease introduced from outside. It appears also that it is usually introduced when there is fairly close contact indoors between patients and normal subjects. There was suggestive evidence that these presumably viral infections precipitated attacks of asthma in susceptible subjects from time to time.

The epidemics resemble that reported by Paul & Freese (1933) in Spitzbergen, where the infection was introduced in spring when the first ship arrived.

On the whole there is also agreement between our observations and those made on small groups of Antarctic explorers, in whom epidemics are introduced from outside although there have also been reports of epidemics arising within these groups, possibly as a result of unpacking clothes (T. R. Allen, personal communication), months after contact with the outside world was broken. Such epidemics are uncommon, however, and possible causative viruses of colds in Antarctic explorers have still not been isolated in spite of serological and other studies (Cameron & Moore, 1968).

We would now like to have further information about the causation of epidemics. Sera collected before the evacuation contained antibodies against parainfluenza viruses, two rhinoviruses and respiratory syncytial virus (Taylor-Robinson & Tyrrell, 1963; J. E. Acornley, personal communication). There was also evidence that they had been infected with influenza viruses (Tyrrell, Peto & King, 1967), although it is unlikely that such a virus caused the epidemics seen in the period of this study. On leaving Britain to return to the island they had more circulating antibody against influenza and parainfluenza than when they were evacuated (Tyrrell *et al.* 1967), nevertheless, it was not long before viruses were being introduced and causing epidemics in the population.

We wish to acknowledge the encouragement of the Tristan da Cunha Working Party of the Medical Research Council.

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The British reference preparation for influenza virus haemagglutinin

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SUMMARY

The two methods for measuring the haemagglutinin content of an influenza virus suspension are the haemagglutinating (HA) and chick cell agglutinating (CCA) techniques and both measure the same biological activity. With the establishment of an international reference preparation for influenza virus haemagglutinin (type A), however, it seems logical to express the haemagglutinin content of influenza vaccines in international units. Accordingly a collaborative study was arranged in order to obtain agreement on the number of units to be assigned to a British reference preparation for influenza haemagglutinin. It was agreed that the preparation contains 190 i.u. per ampoule and 1 i.u. is contained in 0·0622 mg. of the dried material.

INTRODUCTION

It is agreed that there is a correlation between the haemagglutinin content of whole virus influenza vaccines and the ability of the vaccine to protect against the disease, but for many years there has been a controversy over the most accurate method of measuring the potency of influenza vaccine. The haemagglutinin content, which has been taken as an index of potency, has been expressed either as haemagglutinating activity (HA) or chick cell agglutinating activity (CCA). Both measure the ability of the virus to agglutinate red blood cells of the chicken but the absolute values of the activity differ by the two methods.

The World Health Organization (Report, 1968) established an International Unit of influenza haemagglutinin activity and it seemed logical therefore to start expressing the potency of influenza vaccine in International Units rather than in HA or CCA. The World Health Organization International Laboratory for Biological Standardization in Copenhagen has now arranged two series of collaborative assays on a large number of influenza virus vaccines and the results of these studies are soon to be published (Krag & Weis Bentzon, 1971). In order to calibrate a proposed British Reference Preparation in International Units, therefore, a collaborative study was arranged and the purpose of this report is to present the findings and establish the Reference Preparation.

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DESIGN OF THE STUDY

The laboratories and work programme

Four laboratories took part in the study, three of them determined the activity of the vaccines by the HA and three of them by the CCA method.

Each laboratory was requested to determine the activity of five coded preparations A to E in International Units by comparison with the International Reference Preparation and to obtain a ratio of activity for each preparation with the proposed British Reference Preparation. The laboratories were also asked to include in each titration their own working reference preparation.

On each of 3 working days each coded preparation and the proposed British Reference Preparation were titrated in parallel using dilution steps differing by about 30%. On a fourth working day the titrations were repeated but on this occasion the International Reference Preparation was included which was known to contain 200 International Units per ampoule. The International Reference Preparation was to be used in accordance with the recommendations of the World Health Organization collaborative assay of 1968.

The vaccines

The following vaccines were distributed:

Vaccine A. Allantoic fluid containing A_2/ENG/76/66, centrifuged and distributed in 1 ml. volumes in ampoules. Snap frozen and stored at −70° C.

Vaccine B. Allantoic fluid containing B/ENG/5/66, centrifuged and distributed in 1 ml. volumes in ampoules. Snap frozen and stored at −70° C.

Vaccine C. The proposed British Reference Preparation reconstituted with distilled water and distributed in 1 ml. volumes in ampoules. Snap frozen and stored at −70° C.

Vaccine D. Vaccine C diluted 1/4 in distilled water was distributed in 1 ml. volumes in ampoules. Snap frozen and stored at −70° C.

Vaccine E. Normal allantoic fluid from 10-day-old embryonated hen's eggs centrifuged and distributed in 1 ml. volumes in ampoules. Snap frozen and stored at −70° C.

Proposed British Reference Preparation. Distributed as a freeze-dried preparation.

International Reference Preparation. Distributed as a freeze-dried preparation and containing 200 i.u./ampoule.

Before despatch the vaccines were transferred from the −70° C. refrigerator and packed into expanded polystyrene boxes containing solid carbon dioxide. Laboratories 1 and 2 received the vaccines still frozen and they were stored at −60° C. Laboratories 3 and 4 received the vaccines thawed but cold. Laboratory 3 refroze the vaccines at −70° C. whereas Laboratory 4 stored the vaccines at +4 °C. before starting the assays.

ASSAY SYSTEMS

Haemagglutination titrations

The haemagglutinin content was titrated by the method recommended by the World Health Organization (1953).

Diluted preparations were titrated in WHO pattern perspex plates using two-fold dilutions in 0·25 ml. of M/100 phosphate buffered saline, pH 7·2. To each cup 0·25 ml. of an 0·5 % chick erythrocyte suspension were added, and the end point read as that dilution giving 50 % haemagglutination according to the pattern method. The end point of activity was read after standing for 1 hr. at room temperature (20–22° C.) and where the end point fell between dilutions, it was calculated by interpolation.

Erythrocyte suspensions were standardized by colorimetric or densitometric methods, according to the routine practice in each laboratory. A pool of erythrocytes was made from several fowls and used for from 1 to 3 days.

All preparations were titrated in parallel on the same day.

Chick cell agglutination estimations (densitometric readings)

The CCA estimations were based on the original principles of Hirst and Pickles (1942) and modified by Miller (1965) which eliminates the subjective reading of the pattern test and substitutes an objective reading of optical densities (O.D.).

For these estimations, different types of colorimeter or densitometer were used in the different laboratories. The 50 % end-point was determined graphically or by calculation as that dilution lying half way between the O.D. reading of a standard suspension of chick red cells and the O.D. reading of a maximally agglutinated standard suspension of chick red cells.

RESULTS

Haemagglutination titrations

The statistical analysis of the results obtained by measuring the haemagglutinin activity of the virus preparations is shown in Tables 1 to 3 and Fig. 1.

Preparations A, B, C and D were compared with the proposed British Reference Preparation and since it was known that preparation E was a control preparation containing normal allantoic fluid, it has been excluded from any statistical analysis.

In Table 1 the details of the assays comparing preparations A, B, C, D, the proposed British Reference Preparation and the International Reference Preparation are shown. In addition Laboratory 1 included two of their own preparations and these have also been included in the results.

Potency ratios of preparations A, B, C and D were estimated in terms of the proposed British Reference Preparation using the log titres for each preparation measured on the same day or, in the case of Laboratory 1, log titres for the preparations in the assays in which the same chick erythrocytes were used. The variance of each potency was calculated from the pooled variance between log titres of each preparation assayed at the same time and the weights (reciprocal of the variance) were estimated.

When tests for homogeneity of the log potencies of each preparation from each laboratory were carried out it was found that the only estimates of log potency that were homogeneous within a laboratory were preparation D from Laboratories 2 and 3 and preparation A from Laboratory 1. Thus unweighted geometric mean potencies and confidence limits based on the direct estimate of variance of the

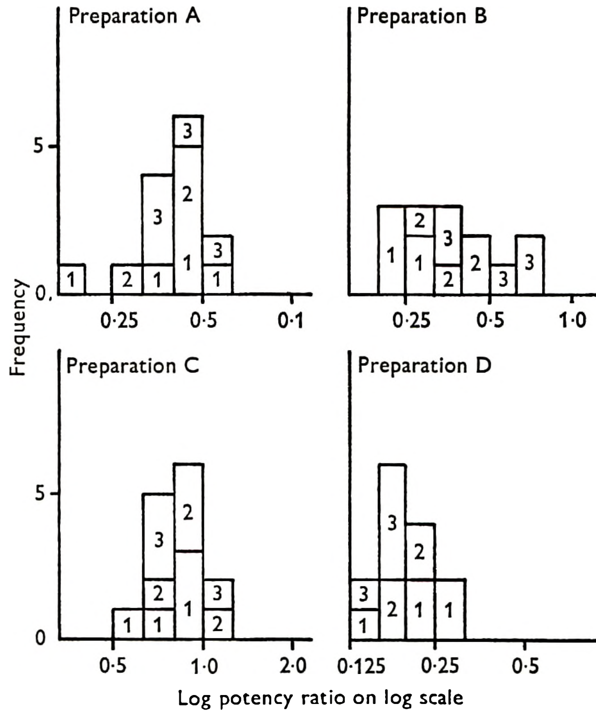

Fig. 1. Frequency distribution of log potency ratios of preparations A, B, C and D in terms of the proposed British Reference Preparation (HA method)

Table 1. *Details of the thirteen assays by the HA test method included in the analysis*

Laboratory number	Preparations compared	No. of assays
1	A, B, C, D, BRP 2 lab. preps.	3
	A, B, C, D, BRP IRP, 2 lab. preps.	2
2	A, B, C, D, BRP	3
	A, B, C, D, BRP IRP	1
3	A, B, C, D, BRP	3
	A, B, C, D, BRP IRP	1

BRP = Proposed British Reference Preparation.
IRP = International Reference Preparation.
Lab. preps. = various laboratory preparations.

log potencies have been computed for each of the four preparations from the three laboratories. These results are shown in Table 2 and frequency distributions of the log potencies, one for each preparation, are given in Fig. 1.

Table 2. *Potency ratios of preparations in terms of the proposed British Reference Preparation by the HA test method*

Preparation	Laboratory 1	Laboratory 2	Laboratory 3	Combined results
A	0·375 0·219–0·640	0·390 0·280–0·542	0·409 0·328–0·510	0·391 0·333–0·460
B	0·245 0·221–0·271	0·364 0·249–0·534	0·503 0·351–0·720	0·354 0·285–0·440
C	0·751 0·554–1·018	0·846 0·624–1·146	1·017 0·563–1·835	0·804 0·716–0·903
D	0·227 0·158–0·328	0·197 0·166–0·240	0·168 0·149–0·190	0·196 0·172–0·223
X*	0·207 0·175–0·245	—	—	—
Y*	8·199 7·216–9·315	—	—	—

Values given as geometric mean potency ratios and 95 % confidence limits based on a direct estimate of variance of the log potency ratios.
* Preparations from Lab. 1.

Table 3. *Potency of the proposed British Reference Preparation in terms of the International Reference Preparation by the HA test method*

Laboratory number	Potency ratio	Overall unweighted mean potency ratio and 95 % confidence limits	Potency i.u. per ampoule
1	0·950 0·680	—	190 136
2	1·197	0·946 0·645–1·387	239
3	1·037	—	207

Preparation C, which was the reconstituted and refrozen proposed British Reference Preparation, should have had a ratio of 1 with the proposed British Reference and the results agree quite well with this. It is also interesting to note that preparation D, which was a 1/4 dilution of C, has indeed been shown to have a ratio of about 1:4 to the proposed British Reference Preparation.

The only preparation for which the variability between the mean log potencies from the three laboratories is significantly greater ($0{\cdot}01 > p > 0{\cdot}001$) than the variability within the laboratories is preparation B. The overall unweighted geometric mean potency ratios and confidence limits are also given in Table 2 and the results of the two additional preparations assayed by Laboratory 1 have been included.

There were only four occasions when the proposed British Reference Preparation and the International Reference Preparation were compared at the same time and so within laboratory variability of the log potencies could not be examined. Weights for the log potencies were estimated in the same manner as described above, but the test for homogeneity showed the log potencies to be heterogeneous and so an overall unweighted mean potency ratio has been computed together with confidence limits based on the direct estimate of variance. The individual potencies and the overall results are given in Table 3.

Chick cell agglutination assays (densitometric method)

The results of the statistical analysis of the CCA assays for preparations A, B, C and D in terms of the proposed British Reference Preparation are shown in Tables 4–6 and Fig. 2.

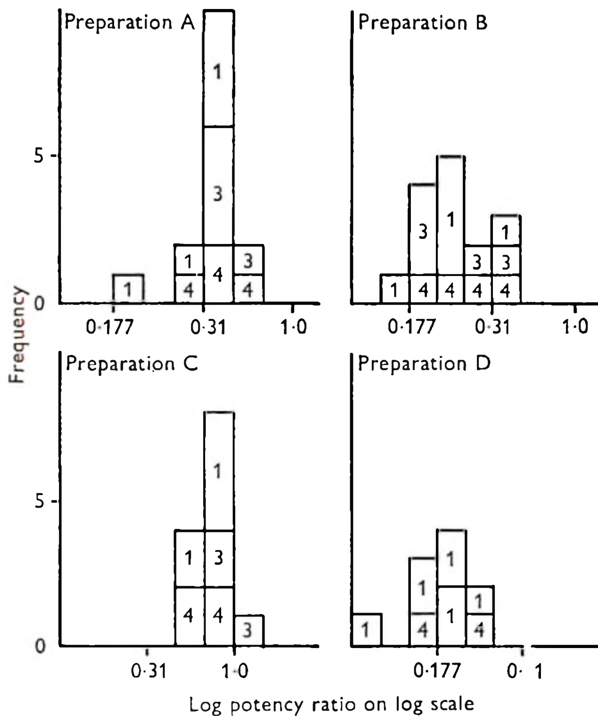

Fig. 2. Frequency distribution of log potency ratios of preparations A, B, C and D in terms of the proposed British Reference Preparation (CCA method)

All CCA assays have been analysed as parallel line assays (Finney, 1952). Each log dose response line was examined by eye and statistical analysis carried out on the data taken from the linear portion of it, omitting the data from the doses that gave no response or a maximum response. Thus the reader may not necessarily obtain exactly the same results by comparing end points of titrations.

In Table 4 the details of the assays comparing preparations A, B, C, D, the proposed British Reference Preparation and the International Reference Prepara-

tion are shown. In addition each laboratory used its own laboratory preparation as a house standard.

Potency ratios of preparations A, B, C and D were estimated in terms of the proposed British Reference Preparation using the log titres measured on the same day or, in the case of laboratory 1, log titres for the preparations in the assays in which the same chick erythrocytes were used. For the same reasons as given for the results from the HA assays, confidence limits based on the direct estimates of variance of the log potencies have been computed and all results have been

Table 4. *Details of the fifteen assays by the CCA test method included in the analysis*

Laboratory number	Preparations compared	No. of assays
1	A, B, C, D, BRP, 2 lab. preps.	4
	A, B, C, D, BRP, IRP, 2 lab. preps.	2
3	A, B, C, BRP, lab. prep.	3
	A, B, BRP, lab. prep.	1
	A, B, BRP, IRP, lab. prep.	1
4	A, B, C, D, BRP, lab. prep.	3
	A, B, C, D, BRP, lab. prep. IRP	1

BRP = Proposed British Reference Preparation.
IRP = International Reference Preparation.
Lab. prep. = various laboratory preparations.

Table 5. *Potency ratios of preparations in terms of proposed British Reference Preparation by the CCA test method*

Preparation	Laboratory 1	Laboratory 3†	Laboratory 4†	Combined potency ratio
A	0·301 0·226–0·400	0·384 0·327–0·450	0·371 0·273–0·505	0·345 0·304–0·399
B	0·244 0·191–0·311	0·241 0·173–0·337	0·251 0·182–0·345	0·245 0·214–0·279
C	0·769 0·638–0·927	0·866 0·622–1·210	0·704 0·572–0·866	0·769 0·694–0·858
D	0·177 0·135–0·232	—	0·199 0·158–0·251	0·186 0·159–0·221
X* Lab. standards	0·237 0·177–0·317	2·171 1·519–3·102	1·821 1·598–2·075	—
Y*	7·617 6·058–9·557	—	—	—

Values given are geometric mean potency ratios and 95% confidence limits based on a direct estimate of variance of the log potency ratios.

* Various laboratory preparations.

† Mean and range.

presented in the same manner. The results are shown in Table 5 and the frequency distribution of the log potencies for each preparation are shown in Fig. 2. Again the results show that the ratio of preparation C to the proposed British Reference Preparation approximates to unity and that the ratio of D is about 1:4.

As with the HA assay method, there were only four occasions when the proposed British Reference Preparation and the International Reference Preparation were compared at the same time and so within laboratory variability of the log potencies could not be examined. The overall unweighted mean potency ratios and ranges however, are shown in Table 6.

Table 6. *Potency of the proposed British Reference Preparation in terms of the International Reference Preparation by the CCA test method*

Laboratory	Potency ratio	Overall unweighted mean potency ratio and range	Potency i.u. per ampoule
1	0·948	0·905	189
3	1·041	0·718–1·040	208
4	0·718		143

CONCLUSIONS

The collaborative assay of five unknown virus preparations by four laboratories using two methods of assay (the HA and CCA) has shown that the absolute values of haemagglutinating activity obtained in any laboratory at any time are dependent upon many factors such as the method of test, and the quality and quantity of the red blood cells used in the test. The results are much more uniform, however, when a reference preparation to which a unit has been assigned is included in each test.

From the results of the HA tests it was shown that the British Reference Preparation had an estimated potency of 189·2 International Units per ampoule with confidence limits of 129·0–277·4 i.u. and the CCA results were in agreement with this.

The British Reference Preparation 64/13 for haemagglutination, which is a stable dried preparation of influenza virus with ampoule to ampoule uniformity, therefore, has been assigned a potency of 190 i.u. per ampoule. The dried weight in each ampoule is 11·82 mg. (range 11·59 to 12·08 mg) and therefore, 1 i.u. is contained in 0·0622 mg.

It would be meaningless to assign an HA or CCA unit in terms of International Units since these ratios differ depending upon the test system and the laboratory doing the test.

We are grateful to Beecham Research Laboratories, Evans Medical Ltd. and Philips Duphar for taking part in the collaborative assay.

Evans Medical Ltd. very kindly supplied the virus suspension for the British Reference Preparation, which was dried by the Biological Standards Division of the National Institute for Medical Research.

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Mosquito-borne infections in Fiji

I. Filariasis in northern Fiji: epidemiological evidence regarding factors influencing the prevalence of microfilaraemia of *Wuchereria bancrofti* infections*

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SUMMARY

A survey of microfilaraemia among the population of Vanua Levu, Taveuni and Koro islands in northern Fiji was conducted in 1968 and 1969 as a prelude to a campaign of mass treatment with diethylcarbamazine.

The prevalences of microfilaraemia were found in the more moist conditions of Taveuni and Koro and on the windward southern side of Vanua Levu to be higher than on the drier northern side of Vanua Levu. On both sides of Vanua Levu prevalences were lower inland than near the coast.

Under apparently similar environmental conditions those of Fijian ethnic origin exhibited a higher prevalence of microfilaraemia than that shown by Indians. This ethnic difference and a difference between the prevalences in male and female Fijians are considered to be due more to higher rates of recovery from microfilaraemia in Indians and Fijian women than to diminished exposure to mosquitoes. Mathematical models have been used as an aid to the interpretation of the data, and, where appropriate, comparison has been made with the prevalence of antibodies to dengue, an arbovirus having the same vectors.

Household infections were analysed by computer techniques. Infections in large households were not proportionately higher than in small households, indicating that transmission was not intrafamilial. The clustering of infections within households, though present, was not marked. Among the occupants of outlying settlements the prevalence of microfilaraemia was relatively low indicating a lower risk of infection due to isolation.

INTRODUCTION

Signs of filariasis have been recognized in Oceania since the times of Captain Cook over two centuries ago and a number of careful surveys have been made both of the disease in man and of infections in the vector mosquitoes. The distribution of

* Much of the information in this paper has been abstracted from an unpublished report to the Director of Medical Services Fiji, 'Filariasis and arbovirus survey, Northern Fiji, 1968–9', by Mataika, Dando & Macnamara (1970).

the disease due to aperiodic *Wuchereria bancrofti* before effective control measures were instituted is known for various island groups, and Iyengar (1965) has given a good review of the epidemiology of filariasis in the South Pacific. Most of the islands in this region are too small to show appreciable internal climatic variations, and their populations are frequently too small to permit subdivisions according to attributes which might influence the epidemiology of filariasis.

The territory of Fiji has the greatest range of climatic differences and the largest population of any island group in the zone of aperiodic filariasis. Only New Caledonia has similar climatic variations, but New Caledonia differs from the others in that the main insect vector is *Aëdes* (*Ochlerotatus*) *vigilax* rather than mosquitoes of the *Aë.* (*Stegomyia*) *scutellaris* group.

The population of Fiji is nearly equally divided between those of Fijian origin and those of Indian origin. The Indian group originally came mainly from the United Provinces and Madras. The Fijians are primarily Melanesian with a Polynesian admixture. In both groups the great majority have been born in Fiji and have grown up there. There is also a relatively small group of Chinese, Europeans, and those of mixed ethnic origin.

The principal mosquito vectors of *Wuchereria bancrofti* in Fiji are *Aë.* (*Stegomyia*) *polynesiensis*, Marks 1951, and *Aë. S. pseudoscutellaris*, Theobald 1910. Other vectors have been incriminated, but are of local or minor importance (Symes, 1960; Burnett, 1960*a*). These two Aëdes mosquitoes were probably also the main vectors of dengue virus which ravaged the area during epidemics in 1930 and 1943 (Maguire *et al.* 1971).

Control of the insect vectors presents considerable obstacles (Burnett, 1960*b*), but control of the parasite by diethylcarbamazine administered to the human population has offered hope in Fiji (Burnett & Mataika, 1964) as it has elsewhere in the Pacific region (Laigret, Kessel, Bambridge & Adams, 1966; Ciferri, Siliga, Long & Kessel, 1969). Hence it was decided in 1966 by the Medical Department of the Government of Fiji that a campaign should be instigated for the control of filariasis by the mass administration of diethylcarbamazine. Initially the campaign was to cover the island of Vanua Levu, south of the main divide, the island of Teveuni and adjacent islets. Later Koro island was added to the area as well as Rotuma island. Nevertheless, before administering the drug, a survey of filariasis was called for, to provide a starting point from which the progress of the campaign could later be evaluated, and to provide, amongst other details necessary for the administration of the campaign, information on the current epidemiology of filariasis.

The survey began in December 1967, but was later extended to areas beyond those mentioned above, owing to the very satisfactory manner in which the control campaign was found to progress.

This paper outlines the procedures employed in the survey, and presents data associating the prevalence of microfilaraemia with climate, ethnic groups, sex and age groups, households and housing density.

A concomitant survey of antibodies against dengue arbovirus was undertaken. The full results of this survey are presented elsewhere (Maguire *et al.* 1971)

but reference will be made to the results where they facilitate the understanding of the epidemiology of filariasis. In Fiji the two diseases are comparable in that (1) man is the sole vertebrate host, (2) the same species of mosquito transmit both diseases (Rosen, Rosebook, Sweet & Sabin, 1954) and (3) the extrinsic incubation periods of the two diseases are similar.

Climatological and social factors

Vanua Levu is the second largest island of the Fiji group. It is approximately 110 miles long and has an average width of 20 miles. A chain of hills attaining a height of 2000 feet forms a backbone of the island. The hills lie across the flow of the prevailing SE trade winds so that on the windward side there is a high annual rainfall with an average of just under 100 in. (2·54 m.) on the coast increasing to about 200 in. inland. Seasonal variations are slight. On the leeward side of the island rainfall is less with an average of about 80 in. (2·03 m.) per annum along the coast, and seasonal variations are more conspicuous with a relatively dry period during the cooler months of the year.

The climatic differences between the two areas are reflected in the vegetation. On the windward side the area is heavily wooded and coconut plantations are common. On the leeward side hill forest rapidly gives way to extensive grassland and the cultivation of sugar cane. Mangrove swamps are more extensive along the northern shores than on the southern more steeply sloping coastline.

The island of Taveuni is smaller than Vanua Levu being 26 miles long and 6–7 miles wide. Although a high chain of hills runs down the middle of the island both sides are humid with high rainfall. Coconut plantations are extensive and the proportion of Fijians living outside villages is higher here than on Vanua Levu.

Koro island is situated to the south of Vanua Levu. It is smaller than Taveuni, but otherwise similar in its climate.

The Fijian* population for the most part lives in village communities. Villages have populations of about 100, and are relatively well kept. In recent years there has been an increasing tendency to break away from village life and for individuals to build their homes near their farms and in relative isolation. Such dwellings are referred to as settlements.

Infants are usually well clothed and kept indoors. From 2 years to 6 years of age, the children roam the village and surrounding areas, often very lightly clad. From 6 to 14 years of age most children attend school.

Adult men are engaged mainly in agricultural work and copra production. The women, when not engaged in their domestic duties, spend most of their time fishing on the reef. Most villages are close to the sea, but women even from inland villages will make frequent visits to the coast.

The dwellings of Indians are usually relatively scattered, although on some copra plantations there are quarters provided for the workers and their families. Most of the cultivation of sugar cane is undertaken by Indians and is concentrated

* The description Fijian or Indian applied to persons refers to their ethnic origin and has no significance in regard to citizenship or nationality. The majority of the population whether ethnically Fijian or Indian were born in Fiji.

on the north side of Vanua Levu. The dwellings are scattered around the cane fields.

In the copra-producing areas the Indian men are largely occupied on the plantations, and the women are mainly engaged in domestic duties. The women are well covered with clothing. In the sugar producing areas women frequently assist on the plantations. Boys and girls when not at school assist their fathers or mothers.

SURVEY METHODS

Two surveys are reported below. The first survey was made on Taveuni island, in southern Vanua Levu and on Koro island. It started early in 1968 and was completed in about 1 year. The second survey was in northern Vanua Levu and was undertaken in June 1969.

Selection of villages for survey for microfilaraemia

First survey. From the report on the census of the population 1966 (Zwart, 1968) was made a list of villages and census areas designated 'Remainder of area'. These were given serial numbers and then selected partly at random and partly by deliberate choice. Forty villages including census areas termed 'Remainder of area' were finally designated for survey. Thirty-seven had been selected at random and three by deliberate choice to cover areas otherwise very poorly represented. The population to be covered by blood examination represented about 15 % of the total in the survey area.

Second survey. In northern Vanua Levu four inland and four coastal villages as well as four small offshore islands were selected for survey by deliberate choice. The inhabitants of these villages were nearly all Fijians. In addition some Indian children from sugar-cane growing areas were examined.

Operational procedures

A survey team of nine persons was formed. This team was responsible for the survey here reported, for an entomological survey, and for a census of the population preparatory to mass treatment. The procedure was to send one member ahead of the main team by 1 or 2 days to explain to the inhabitants of the village the aims of the work. He would then number all the houses in the village and prepare lists of all houseowners and occupants of the houses. The main team would arrive the following day and would see and examine by households all persons present.

Blood examination for microfilariae

The survey was conducted during daylight. As far as possible venous blood was collected from everyone but when this was not possible capillary blood was usually obtained, although on the offshore islands of northern Vanua Levu and for Indian school children in northern Vanua Levu only capillary blood was examined. Three thick smears each of 20 mm.^{3} blood were made, dried and later stained with

Giemsa stain. The smears were examined for microfilariae under a microscope, often a 'Visopan' projection microscope, and the number of microfilariae in each drop was recorded. If a smear was unsatisfactory, it was ignored and recorded as not examined. The arithmetic mean number of microfilariae per 20 mm.^{3} drop was the number taken for all analyses.

RESULTS

Survey coverage

First survey. The blood examination of two villages had to be ignored as the smears were spoiled by rain.

Of the total numbers of Fijians and Indians in the villages and areas selected for survey the percentages who were examined for microfilaraemia were 68 and 55 respectively. Losses from the desired 100 % coverage were largely in the regions designated 'Remainder of area' and in the 0 to 4 year old age group. Coverage in the typical 'Fijian village' was over 80 %. In these villages the losses were primarily due to temporary absence and rarely due to wilful refusal to be examined, since the co-operation of the people was excellent. Although temporary visitors to a village were examined, they were not recorded as inhabitants of the village; and, unless they happened to be permanent inhabitants of one of the other villages which were to be surveyed, their records would not have been analysed.

Second survey. The coverage of the Fijian villages was comparable to that of the first survey. The 256 Indian school children came from homes in and around Labasa.

Prevalence of microfilaraemia

An initial examination of the results indicated that there were differences in prevalence in the geographical areas of Taveuni and Koro, southern Vanua Levu, and northern Vanua Levu. The entomological work had shown that there were vector differences between villages situated one half mile or less from the sea and those situated further inland. Therefore data from villages situated in these two different areas were analysed separately. Data on persons living on estates or in settlements were first analysed separately, but here the results were similar to those of inland villages, although a high proportion of this population were living close to the sea. For Fijians the results derived from inhabitants of inland villages, settlements and estates have been combined. Since Indians do not live in villages, the results concerning them apply to settlements and estates only. In northern Vanua Levu there was little difference between the results from offshore islets and coastal villages. The results have been combined.

In Tables 1–3 are presented prevalences of microfilaraemia divided according to geographical region, race and sex, with subdivisions according to age, where justified. Also presented are the geometric mean counts of microfilariae per 20 mm.^{3} blood of positive cases. This figure is almost identical with the median count and will be referred to as the Mf. D_{50}. The standard deviation of the logarithm of the counts is expressed as the logarithm to the base 10. The reciprocal of this figure is equivalent to the factor '*b*' described in the analytical procedures of the Expert Committee on Filariasis (W.H.O. Expert Committee, 1967).

Table 1. *Prevalence of microfilaraemia in Fijian males*, 1968–9

	Taveuni Koro	Vanua Levu: Southern coastal	Vanua Levu: Southern inland and settlements	Vanua Levu: Northern coastal and islets	Vanua Levu: Northern inland
No. of villages/districts ...	8	16	14	10	4
Age (yr.)					
0–< 5	77* (0)†	96 (0)	72 (0)	89 (0)	21 (5)
5–< 10	87 (13)	112 (1)	69 (0)	109 (1)	35 (3)
10–< 15	63 (19)	84 (6)	45 (4)	83 (7)	24 (4)
15–< 20	35 (29)	58 (16)	34 (9)	48 (6)	16 (0)
20–< 30	64 (53)	78 (35)	39 (21)	48 (12)	30 (3)
30–< 40	56 (41)	90 (46)	40 (28)	54 (18)	25 (12)
40–< 50	38 (32)	56 (46)	36 (25)	39 (28)	16 (25)
50–< 60	39 (46)	35 (40)	16 (28)	50 (28)	19 (5)
⩾ 60	24 (27)	27 (41)	15 (67)	26 (27)	
All ages	483 (27)	636 (21)	366 (13)	549 (10)	186 (6)
Positive cases: G.M.‡	14	10	9	4	2
Log_{10} S.D.	0·627	0·633	0·640	0·606	0·704

* Number of individuals examined.
† Percentage of individuals showing microfilaria, 0·3 per 20 mm.^{3} of blood.
‡ G.M. = geometric mean of number of microfilariae in 20 mm.^{3} of blood.
Log_{10} S.D. = standard deviation of log_{10} of individual microfilarial counts.

Table 2. *Prevalence of microfilaraemia in Fijian females*, 1968–9

	Taveuni Koro	Vanua Levu: Southern coastal	Vanua Levu: Southern inland and settlements	Vanua Levu: Northern coastal and islets	Vanua Levu: Northern inland
No. of villages/districts ...	8	16	14	10	4
Age (yr.)					
0–< 5	71 (0)	112 (2)	73 (0)	63 (2)	21 (0)
5–< 10	93 (5)	114 (7)	49 (0)	97 (4)	32 (0)
10–< 15	64 (14)	92 (7)	42 (5)	66 (1)	23 (4)
15–< 20	36 (28)	49 (18)	30 (13)	50 (14)	15 (0)
20–< 30	70 (26)	108 (30)	75 (11)	62 (8)	30 (3)
30–< 40	52 (27)	85 (21)	66 (18)	68 (7)	34 (6)
40–< 50	41 (39)	56 (29)	37 (22)	52 (8)	19 (0)
50–< 60	21 (38)	41 (37)	19 (42)	30 (10)	21 (10)
⩾ 60	16 (38)	31 (42)	15 (47)	22 (14)	
All ages	464 (19)	688 (17)	406 (12)	510 (6)	195 (3)
Positive cases: G.M.	5·5	4·1	4·4	3·4	1·0
Log_{10} S.D.	0·859	0·662	0·596	0·584	—

For explanation, see Table 1.

Infections in households

For the areas Taveuni and Koro islands, coastal southern Vanua Levu, and inland southern Vanua Levu, Fijian households were analysed according to the number of occupants and the number of these who showed microfilaraemia. Households of more than 10 were excluded. From the data obtained, a figure,

the mean infected proportion of the household, was derived by dividing the mean number of microfilaraemic individuals in a household of given occupancy by the number of these occupants. These figures are comparable between households of different sizes. The results are shown in Table 5.

Table 3. *Prevalence of microfilaraemia and microfilarial density among Indians and 'other races' of all ages*

Area	Race	Males No. ex.	Males % +	Males Mf. D. geom. mean	Females No. ex.	Females % +	Females Mf. D. geom. mean
Taveuni	Indian	130	11	26	141	14	9
S. Vanua	Indian	160	10	4	156	3	3
Levu	Other	104	18	4	100	10	4
N. Vanua Levu	Indian (Age 15–22)	189	0	/	67	1	/

Table 4. *Infection (a) and recovery (b) rates indicated by microfilaraemia among Fijians in three different epidemiological areas in northern Fiji and used to construct mathematical models*

Sex		Area: Taveuni and Koro	Area: Southern Vanua Levu: Coastal	Area: Southern Vanua Levu: Inland
Both M. and F.	Infection rate	0·085	0·080	0·044
M.	Age	15+	15+	15+
	Recovery rate	0·100	0·110	0·119
	Asymptote of Mf. prevalence	0·46	0·42	0·27
F.	Age	7·5–40	7·5–40	10–40
	Recovery rate	0·230	0·240	0·231
	Asymptote of Mf. prevalence	0·27	0·25	0·16

Prevalence $= (1-e^{-(a+b)t})\, a/(a+b)$ where a = infection rate, b = recovery rate, t = years of exposure.

These proportions have been compared with those which might be expected if the infected individuals allowing for risk appropriate to age and sex were distributed at random among households.

The stochastic frequency of infected individuals in households was obtained by computer analysis employing Monte Carlo simulation and the observed probabilities of microfilaraemia pertaining to specific age/sex groups within the area under review. The 'expected' infected proportions of households are shown in Table 5.

In Table 6 are shown the observed and expected distributions of households containing a stated number of infected individuals.

The interpretation of these results will be presented in the discussion.

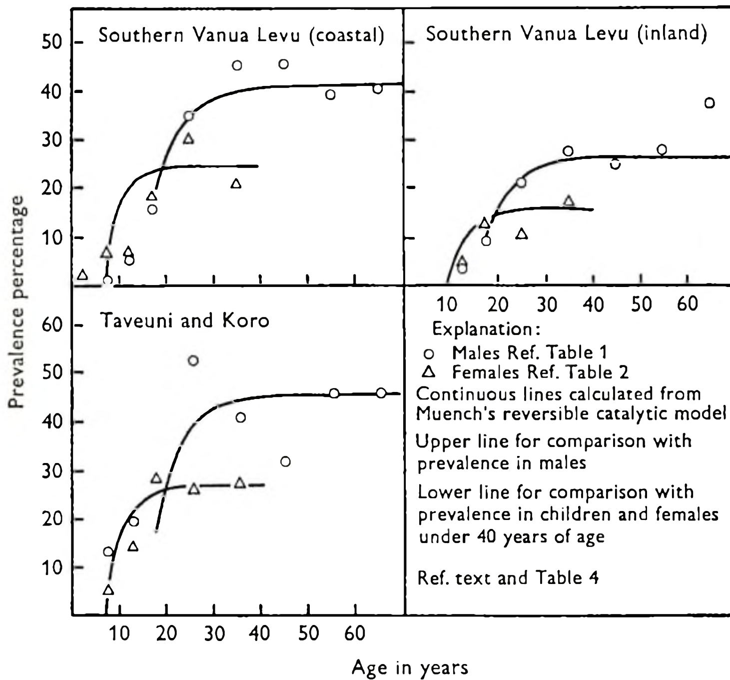

Fig. 1. Prevalence of microfilaraemia.

DISCUSSION

Stability of epidemiological pattern

The population examined, with the exception of those under 5, showed figures for race, sex and age distributions which were close enough to those of the 1966 census to show that no particular selection of individuals had been introduced into the survey.

Although the population of the area had shown considerable natural increase since the previous national census in 1956 (Zwart, 1968), neither immigration nor emigration had been marked.

Surveys for microfilaraemia were conducted in the past in the same areas as our surveys (Nelson & Cruickshank, 1955; Symes, 1960). Burnett (1960*a*) in reviewing these concluded that the epidemiology of filariasis had remained relatively stable for a considerable period. The results of our surveys did not contradict this opinion.

Epidemiological groupings associated with climate and local geography

Symes (1960) pointed out the differences between the prevalence of microfilaraemia among Fijians north of the main divide on Vanua Levu and the prevalence on the southern side. He also noted a lower prevalence in inland villages. The differences, confirmed and more closely defined by our survey, can be attributed to the climate which on the northern side is drier and has a longer season with low rainfall. Whether north or south of the main divide the difference between coastal

Table 5. *Mean expected and observed numbers of microfilaraemic individuals in households of different sizes expressed as proportions of the total number of occupants*

Area		Number of occupants in the household									
		1	2	3	4	5	6	7	8	9	10
Taveuni and Koro	No. households	20	11	16	21	22	23	15	19	12	6
	P. obs.*	0·400	0·318	0·229	0·262	0·209	0·254	0·210	0·211	0·241	0·200
	P. exp.†	0·450	0·318	0·208	0·209	0·218	0·181	0·210	0·237	0·231	0·150
S. Vanua Levu, coastal	No. households	20	26	33	43	44	33	24	31	14	10
	P. obs.	0·400	0·308	0·232	0·209	0·191	0·126	0·155	0·157	0·111	0·150
	P. exp.	0·250	0·385	0·303	0·209	0·177	0·121	0·173	0·153	0·135	0·250
S. Vanua Levu, inland	No. households	11	13	14	13	9	12	17	2	8	4
	P. obs.	0·091	0·115	0·119	0·096	0·200	0·125	0·118	0·062	0·181	0·125
	P. exp.	0·091	0·231	0·095	0·115	0·111	0·153	0·168	0·062	0·152	0·100

* Proportion observed. † Proportion expected from stochastic simulation.

and inland areas can be noted within a mile of the coast. The change is probably due to the decline away from the coast of the numbers of the mosquitoes of the species *Aë. polynesiensis* and their replacement by smaller numbers *Aë. pseudoscutellaris*.

There appears to be little or no transmission among Indians living among the sugar plantations on northern Vanua Levu.

Table 6. *Percentage of total of households containing specified number of individuals with microfilaraemia*

Area	Total no. households		Percent total containing n infected individuals $n = 0$	1	2	3	4	χ^2 P
Taveuni and Koro	165	O	35	32	17	11	5	
		S	27	41	23	8	1	$< 0{\cdot}01$
S. Vanua Levu, coastal	278	O	39	43	11	6	1	
		S	36	39	19	5	1	$< 0{\cdot}01$
S. Vanua Levu, inland	103	O	54	33	9	3	1	
		S	52	33	11	3	1	0·7

O = Observed number. S = Expected number from stochastic frequency.

Differences between ethnic groups

Different prevalences of microfilaraemia in distinct ethnic groups may be easy to demonstrate, but are usually difficult to evaluate owing to numerous and often ill-defined variations in the dress, habits, and customs of the groups. In our survey the prevalence of microfilaraemia was always higher among Fijians than among Indians living in similar geographical and climatic situations: even when the prevalences in the two ethnic groups living in settlements were compared, that among Fijian males was found to be slightly higher than among Indian males. Nevertheless, the fact that the maximum prevalence among Indian males was reached at a young age, the observation that elephantiasis among Indians was more prevalent than among Fijians (Mataika *et al.* 1971) and the report by Maguire *et al.* (1971) that the prevalence of dengue antibodies among Indian males was no less than among Fijian males under comparable circumstances suggest that the lower prevalence in Indians could be due to a higher recovery rate, a situation perhaps similar to that producing the differences between the two sexes in Fijians (see below).

Sex differences

Differences in prevalence between the sexes have been shown in most surveys on Bancroftian filariasis as well as in other filarial infections (McCarthy & Fitzgerald, 1955; Jachowski & Otto, 1955; Beye *et al.* 1963; Crosskey, Crosskey & Macnamara, 1959). These differences have been attributed usually to the different ways of life of the two sexes (McCarthy & Fitzgerald, 1956) although this view has been questioned in some instances. Jordan (1955) suggested that in East Africa women were less susceptible than men to Bancroftian filariasis, but did not

define what was meant by the term susceptible. Moreover, it is known that in experimental infections with some filariae in laboratory animals the female hosts have acquired infection less readily than males (Haley, 1958).

The most marked sex difference was shown in our surveys among Indians on southern Vanua Levu. The difference here may readily be attributed to the differing habits of the two sexes. The women are extensively covered by their clothes and they do not work appreciably in the plantations or farms. Their lower exposure to mosquito-borne disease is also demonstrated by the prevalence of dengue antibodies among them; it being 4% compared with 10% in males (Maguire *et al.* 1971).

Whereas it may be easy to explain the lower prevalence in Fijian women than in men by the greater coverage with clothes and their customs of either working within the village or fishing on the reef, this may not be the entire explanation. Women have acquired dengue antibodies at least as frequently as their men folk and presumably from the same species of mosquitoes as those transmitting filariae (Maguire *et al.* 1971). Rates of recovery from filarial infection which were higher in women than in men could result in the observed differences in prevalence of microfilaraemia.

Age differences

The general pattern of age prevalence parallels that of earlier surveys and of surveys elsewhere in the Pacific region. Microfilaraemia was virtually absent among those under 5 years of age. Between 5 and 15 years of age the incidence remained low. From 15 years of age the prevalence increased rapidly reaching a maximum in the 40+ year age groups. Hayashi (1962) and Hairston & Jachowski (1968), regarding the principles presented by Muench (1959), presented mathematical models to elucidate the picture. In agreement with the conclusions of Hairston & Jachowski, we found that Muench's reversible catalytic models were most similar to the observed data. Table 4 presents rates of acquiring infections and rates of recovery for Fijian males over 15 years old, used in deriving mathematical models which in Fig. 1 are compared with the observed data shown above in Tables 1 and 2.

The derivation of models comparable to the observed prevalence rates in females was not simple, since among females the observed data suggest a rise in prevalence after the age of 7·5 years followed by a plateau of prevalence between the ages of 17 and 40 years, whereafter a further rise may be indicated.

Such a plateau of prevalence among females aged 20–40 is not peculiar to our surveys and is discernable in the data of Jachowski & Otto (1955) in American Samoa, of Marshall & Yasukawa (1966) in the Ryukyu islands, and of Sasa, Mitsui & Sato, (1963) in the Amani islands. It is not evident, however, in surveys in Western Samoa nor in the Cook Islands (McCarthy & Fitzgerald, 1956; McCarthy, 1959). If it is assumed that the plateau in the 17–40 age group is a balance between the rate of acquiring infection and the rate of recovery, and that in females the rate of infection is the same as that in males, then recovery rates in females in this age group may be derived. Models have been constructed and are compared with the observed data in Fig. 1, for three areas of different risk of infection. Table 4

presents the parameters used in deriving these models. Nevertheless, it should be realized that, in making comparison between observed data or phenomena and the parameters of the models, the models indicate only average rates, and that there could be wide variations from these averages in individual experiences. Moreover, recovery rates should be related to the production of microfilariae and not necessarily to the average life of the filarial parasite.

Hairston & Jachowski (1968) on analysing their Samoan data have indicated that from their models they would not expect uniform recovery rates owing to clustering of infections. Our models suggest that although there may be clustering, a factor which certainly is not excluded, such clustering does not greatly affect mean recovery rates.

The point of origin of the curves for females is 7·5 years for the two areas, Taveuni/Koro, and coastal southern Vanua Levu. For inland southern Vanua Levu, however, the origin is at 10 years. This delay for the area of lower infection risk can be explained by the need for multiplicity of infections, probably about two, required to induce a recognizable microfilaraemia.

In northern Vanua Levu, among Fijians there appear to be both low rates of acquiring infection and very low rates of recovery. Nevertheless, in this area there is less information on the stability of the population or of the epidemiology, both of which factors might, if irregular, confuse the picture.

Infections in Fijian households in villages

Size of household

The investigation the results of which are shown in Table 5 was designed to determine to what extent transmission of filariasis was intrafamilial; the argument being that, if it were, large households, with a greater chance of one of their numbers introducing the disease, would show a higher prevalence of infection (microfilaraemia) than would small households. This was shown not to be the case, since the proportions of the total occupants in households who were observed to be infected were very close to those which could have been expected by chance in a random distribution of the population of similar age/sex composition and risk of infection.

The conclusion that transmission is not predominantly intrafamilial enhances the opinion that *Culex fatigans*, a peridomestic mosquito in Fiji, is relatively insignificant as a vector in this area, (Burnett, 1960*a*). These findings may be contrasted with those of Omori (1965) in areas where periodic nocturnal Bancroftian filariasis is prevalent.

Clustering of infected households

This investigation was to determine whether some households, irrespective of size, had more or less than their random share of infection. From Table 6 and in Fig. 2 it is seen that on Taveuni and Koro and in coastal villages of southern Vanua Levu, the spread of the observed distribution of households with specified numbers of infected individuals was greater than could be expected, indicating that some houses do have more than their random share of infections while others

go free. Although the differences between observed and expected numbers are statistically significant on Taveuni, and Koro, and in coastal villages of southern Vanua Levu, the differences are by no means large, and in inland villages are not statistically significant. The epidemiological import of the differences is probably slight.

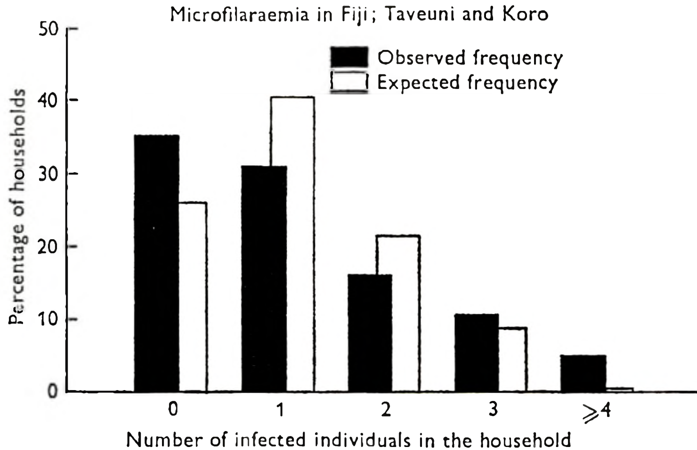

Fig. 2. Household infections.

Effects of housing density

Prevalences of microfilaraemia in Fijian settlements in southern Vanua Levu were lower than in coastal villages, and were so similar to those in inland villages that the results were combined in Tables 1 and 2. Nevertheless, a very high proportion of 'settlements' in southern Vanua Levu are close to the coast and similar in other respects to village houses, except for their degree of isolation. It may be concluded that the existing tendency to establish settlements is beneficial in regard to risk of exposure to filariasis of the inmates.

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Mosquito-borne infections in Fiji

II. Arthropod-borne virus infections

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SUMMARY

Surveys of arbovirus activity in Fiji were conducted over a 10-year period from December 1959 to December 1969. No arboviruses were isolated from over 200,000 mosquitoes, 9000 ticks, or 575 serum samples. Eight thousand human and 1117 bird, bat and animal sera were tested for haemagglutination-inhibiting arbovirus antibody using a variety of group A, group B and Bunyamwera group antigens. Only a small number of low-titre reactions were found among the non-human sera, but 14 % of all human sera were found to contain Group B antibody. The antibody prevalence increased with increasing age, from less than 1 % for persons born since 1950, to 70 % for persons born before 1900. The age differences in prevalence could be used to estimate the time and size of previous epidemics. Differences were found in antibody prevalence between the sexes, between ethnic groups and between persons from different regions. These differences could be explained in terms of climate, location and custom.

Historical and serological evidence both suggest that all the antibody detected was due to past exposure to dengue virus. The very high proportion of the population with no dengue antibody makes Fiji a high-risk area for a further dengue epidemic. Dengue virus is known to be active in the Pacific and South-East Asia.

INTRODUCTION

Periodic epidemics of dengue have occurred in Fiji since the first epidemic reported in 1885. There have been two major outbreaks since then: one in 1930 and the other in 1943–4. Clinical cases of dengue were reported in the intervening periods, suggesting that dengue was endemic in these islands, but in recent years the number of cases notified has declined and since 1955 has not exceeded 40 cases per year. There have been no cases notified since the beginning of 1967.

During the period December 1959 to December 1969 numerous surveys were carried out in order to establish the status of arbovirus infections in the Fiji group. This work included attempted isolation of viruses from ticks, mosquitoes and

human, bird and various animal bloods, together with serological studies on serum samples from humans, animals and birds.

Preliminary results (Miles *et al.* 1964) showed that most, if not all, of the arbovirus activity in Fiji could be attributed to dengue virus. Over the past 10 years dengue, especially haemorrhagic dengue, has become increasingly important in South-East Asia, where it has caused the death of scores of children. The Fiji survey was expanded therefore to learn more of the epidemiology of this virus in Fiji, and to maintain surveillance because of the constant threat of the reintroduction of the virus in an epidemic form. More recently studies have been undertaken to compare the epidemiology of dengue with that of filariasis, which in Fiji is almost certainly carried by the same mosquito vectors (Mataika, Dando, Spears & Macnamara, 1971).

This report describes the results of the survey work and analyses some of the factors involved in the epidemiology of dengue in Fiji.

MATERIALS AND METHODS

Regions surveyed

The surveys conducted up to the end of 1967 covered most of the main regions of Fiji, including coastal and inland Viti Levu, Beqa, Southern Vanua Levu, Northwest Taveuni, the small islands of Qamea and Rabi, Kadavu (a full-scale survey), and Naviti in the Yasawa group. Since some of these surveys were designed primarily to detect recent arbovirus activity, the samples tested came from a higher proportion of children than is found in the total population. The 1968–9 survey was carried out in conjunction with the Fiji Medical Department filariasis

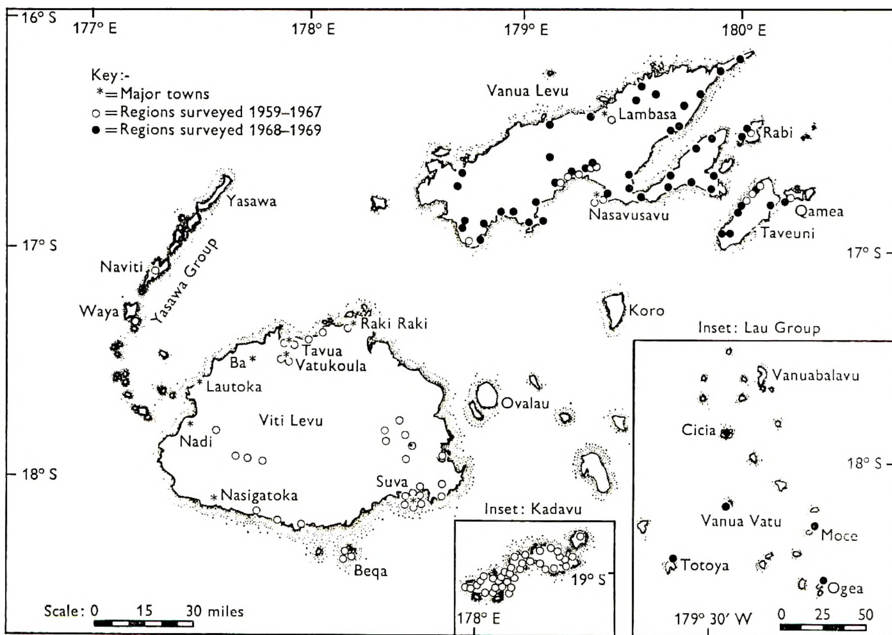

Fig. 1. Arbovirus antibody surveys in Fiji 1959–69.

survey in Northern and Southern Vanua Levu, Taveuni and the Lau Islands. For this survey, the field procedures, sampling methods and climatological and social factors involved in the selection of samples have been described elsewhere (Mataika *et al.* 1971). Detailed recording, punch-card sorting and computer analysis made it possible to gain more information from these collections than from the earlier work. The 1968–9 collections contained over half of the 8000 human sera tested during the total survey period.

The locations of the various regions studied are shown in Fig. 1.

Virus isolation attempts

A total of over 200,000 mosquitoes representing eleven species (but mainly *Aëdes* (*Stegomyia*) *polynesiensis* Marks; *Aëdes* (*Aëdimorphus*) *vexans* Theobald; *Aëdes* (*Finlaya*) *fijiensis* Marks; and *Culex* (*Culex*) *annulirostris* Skuse) and over 9000 ticks representing three species (*Haemaphysalis* (*Kaiseriana*) *longicornus* Neumann; *Rhipicephalus sanguineus*, and *Amblyomma cyprium* Neumann) were tested for virus using the methods described by Miles *et al.* (1964). During the early surveys, insect pools were inoculated into suckling mice, yolk sacs of 6-day embryonated hen eggs, and on chick-embryo cell monolayer tissue cultures, but after 1964 only intracerebral inoculation of suckling mice and inoculation of BHK 21 cell tissue culture tubes were used.

Sera from 140 humans suffering from ill-defined febrile sickness, from 84 fruit and insectivorous bats, and from 351 fowls and other birds were also tested for virus during the survey period.

Serological studies

Sera from 8000 humans, 44 bats, 1062 fowl and other birds and 11 rodents and other animals were tested for arbovirus haemagglutination-inhibiting (HI) antibody using a variety of group A, group B and Bunyamwera group arbovirus antigens. During the first few survey years the sera were tested against 13 or 14 antigens selected from western equine and eastern equine encephalitis (WEE and EEE), Whataroa, Sindbis, Bebaru, Semliki Forest, Chikungunya, Murray Valley encephalitis (MVE), Japanese encephalitis (JE), St Louis encephalitis, yellow fever, dengue types 1 and 2, Cache valley, Ntaya and Batai viruses. During the later years sera were routinely tested against seven antigens only. These were WEE, Whataroa, MVE, JE, dengue 1, dengue 2 and yellow-fever viruses. A set of 44 bat, 36 fowl and 134 human sera was tested against the additional antigens of Getah, Ross River, Tembusu, California encephalitis and Edge Hill viruses.

Antigens were prepared from infected suckling mouse brain by sucrose-acetone extraction as described by Clarke & Casals (1958). Sera routinely were triple-extracted with acetone to remove non-specific inhibitors, then absorbed with excess goose cells to remove non-specific agglutinins. Screening tests were carried out using 4–8 units of antigen and a serum dilution of 1/10. Sera which were positive at this dilution were then titrated against the appropriate antigens. Complete inhibition of 4–8 units of antigen at a dilution of 1/10 or higher was considered a positive reaction.

RESULTS

Virus isolation

No arboviruses were isolated from any of the specimens. Several agents were isolated from mosquitoes and human sera in mice or in tissue cultures (BHK 21) and these were identified as Coxsackie A 6 viruses (Maguire & Macnamara, 1966) and reovirus type 3 (Miles & Stenhouse, 1969) respectively. A Coxsackie A-like virus (TP 275) also was isolated from a pool of *Haemaphysalis longicornus* (A. C. Stenhouse, unpublished observation).

Serology

Five of 44 bat sera were positive at titres of 1/20 or 1/40 against MVE antigen. Each specimen came from a different locality. Four of 36 fowl sera inhibited Getah antigen at a titre of 1/20. All other birds, bats, rodents and domestic animals were negative.

From all areas of Fiji, human serum samples were found which contained arbovirus group B HI antibody. Many, especially those from older persons, reacted broadly to most or all group B antigens, while others reacted with only one group B antigen, usually dengue 1, dengue 2 or MVE. For the purposes of analysis, those sera which reacted only with yellow-fever antigen were classified as negative. This was rare, occurring only with sera from Europeans with a history of yellow-fever vaccination.

Table 1. *Prevalence of arbovirus antibody within the major ethnic groups*

	Fijian			Indian			Other races*			
Sex	+ve	−ve	%+	+ve	−ve	%+	+ve	−ve	%+	Total
Female	502	3039	14·2	25	232	9·7	16	81	16·5	3895
Male	516	3200	13·9	46	232	16·55	26	85	23·4	4105
Total	1018	6239	14·0	71	464	13·3	42	166	20·2	8000

* Includes Europeans, Chinese, other Pacific Islanders and peoples of mixed racial origin.

No human sera were found which reacted with antigens other than Group B. Most positive sera reacted to highest titre with dengue 1 or 2 antigens, and this, taken with the results of dengue neutralization tests reported previously (Miles *et al.* 1964), indicates that practically all the antibody detected could be due to past infection with dengue virus. High titres (up to 1/2560) were found in all age-groups from 20 years of age and upwards. The titres of persons under the age of 20 years were generally much lower. Apart from this there was no correlation between titre and age, sex or race.

Table 1 shows the population tested for arbovirus HI antibody listed according to ethnic group, sex and result of the HI test. There was no difference between the prevalence of group B antibody in Fijian males and females, or between the prevalence in Fijians and Indian males, but the prevalence in Indian females was significantly lower. The total number of specimens from people of other races did

not permit statistical comparison between the sexes, but this group as a whole did have a significantly higher antibody prevalence than the remainder of the survey population.

Table 2. *The effect of age on the prevalence of group B arbovirus antibody*

Year of birth	Positive M	Positive F	Positive Total	Negative M	Negative F	Negative Total	Positive (%)
Before 1900	34	29	63	23	4	27	70
1900–1904	40	28	68	24	9	33	67·3
1905–1909	45	36	81	32	21	53	60·5
1910–1914	70	58	128	54	42	96	57·1
1915–1919	56	62	118	58	42	100	54·1
1920–1924	68	65	133	81	61	142	48·4
1925–1929	69	70	139	91	73	164	45·9
1930–1934	85	68	153	137	120	257	37·3
1935–1940	61	79	140	132	165	297	32·0
1941–1944	29	26	55	177	230	407	11·9
1945–1949	14	10	24	181	255	436	5·2
1950–1954	5	2	7	508	518	1026	0·68
After 1954	12	10	22	2019	1812	3831	0·57
Total	588	543	1131	3517	3352	6869	14·14

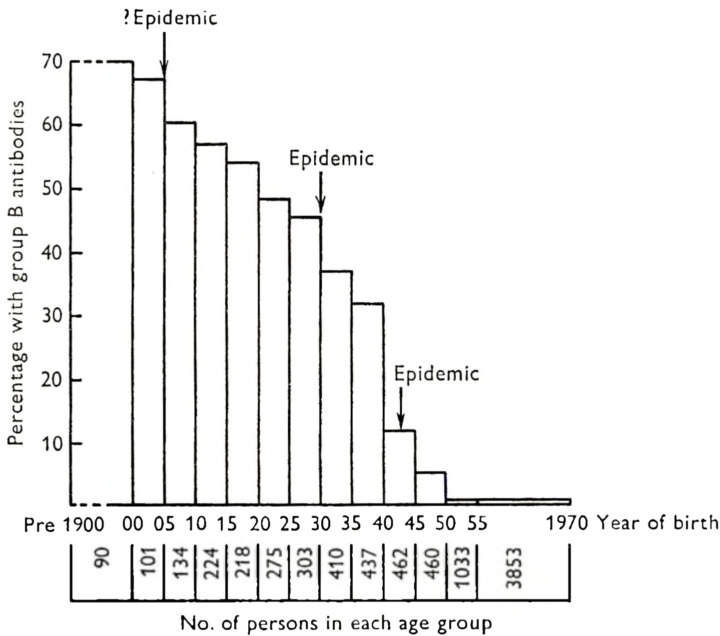

Fig. 2. The effect of age on prevalence of group B antibody.

Table 2 shows antibody prevalence tabulated according to date of birth and sex. The table shows that there is a gradual decline in prevalence with decreasing age and that virus activity must have been virtually absent since 1950. When the totals for males and females born before 1905 are pooled it becomes apparent that there is a significantly higher prevalence in females (81·5 % positive) than

Table 3. *Geographical variation in arbovirus antibody prevalence*

Region	Sex: Male +ve	Male −ve	Male %+	Female +ve	Female −ve	Female %+	Total +ve	Total −ve	Total %+
Northern Vanua Levu									
Inland villages	15	153	8·9	18	156	10·3	33	309	9·65
Coastal villages	40	240	14·3	28	223	11·15	68	463	12·8
Southern Vanua Levu									
Inland villages	32	126	20·25	44	139	24·0	76	265	22·3
Settlements	40	300	11·8	30	309	8·85	70	609	10·3
Coastal villages	89	346	20·45	106	369	22·3	195	715	21·4
Taveuni	132	258	33·9	92	277	24·9	224	535	29·5
Lau Islands	37	350	9·6	72	374	16·15	109	724	13·1
Kadavu	80	770	9·4	106	681	13·5	186	1451	11·4
Viti Levu and remainder of survey area	123	974	11·2	47	824	5·4	170	1798	8·6
Total	588	3517	14·3	543	3352	13·94	1131	6869	14·14

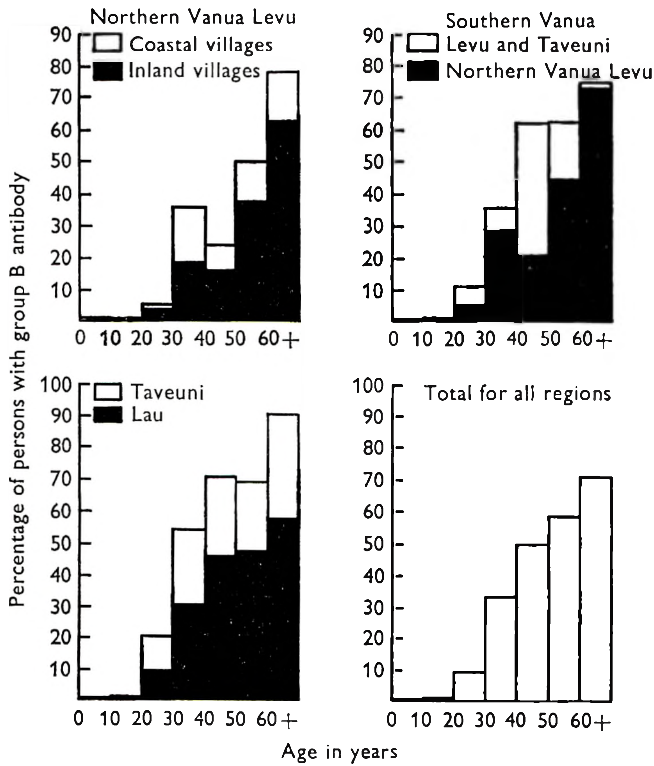

Fig. 3. Antibody prevalence in different geographic regions.

in males (61 % positive). The endemic type of increase in prevalence with age before 1930 and the steps in the incidence rates produced by the 1930 and 1943 epidemics are shown more clearly in Fig. 2.

The variation in antibody prevalence in the various geographical locations is shown in Table 3. Considerable differences were found between areas of differing climate or differing house patterns. As explained above, the rather low prevalence in Viti Levu and the remainder of the survey area can be attributed to the disproportionate numbers of children in these collections, but in all other areas differences in prevalence are real and cannot be explained by differences in the age structures of the populations tested. Some areas showed considerable differences not only in total prevalence, but in prevalences in different age-groups. These differences by region and age-group are shown graphically in Fig. 3.

DISCUSSION

The evidence presented here supports the hypothesis that the only arbovirus which has been active in Fiji is dengue. The low antibody titres in a few bats and fowls probably signify nothing more than non-specific group-reacting substances. The serological evidence that there has been little or no dengue activity since 1950 is supported by the failure to detect virus in any of the material tested. In spite of the lack of recent dengue activity, analysis of the serological results provides a considerable amount of information concerning the epidemiology of dengue in Fiji.

The relationship between race, sex and antibody prevalence

The prevalence of dengue antibody in Fijian males and females, Indian males, and females of 'other races' was essentially the same, but Indian females had a significantly lower prevalence and males of 'other races' had a significantly higher prevalence. The low antibody prevalence in Indian women is readily explained by the fact that they traditionally are extensively covered by clothes and do not work with males on the farms or in the plantations. This lower exposure of Indian women to mosquito bites is reflected in their lower microfilaraemia prevalence. The equal prevalence of dengue antibody in Fijian males and females contrasts with the situation in filariasis, where women are less affected than men (Mataika *et al.* 1971). The reason for the higher prevalence of antibody in males of 'other races' is not clear. Only a small proportion of these men would have come from other regions of known dengue activity.

The Indians of Southern Vanua Levu settlements were found to have lower antibody prevalence (7·1 %) than Fijians from the same area (12·8 %). This difference is probably due to the different housing types. The Indian population in these areas tend to live in concentrated 'barrack'-type houses, whereas the Fijians live in isolated houses. The differences may therefore be due to the better mosquito control in the immediate vicinity of the Indian dwellings.

The differences between the sexes in other regions cannot be explained so readily. In Taveuni there is a significant difference between the sexes, the males having an antibody prevalence of nearly 34 % while the females have a prevalence of only

25 %. This difference was not associated with race ratios in the sample. On the other hand, in Kadavu and Lau the position is reversed. Local custom may play an important role in determining the relative exposure of males and females to mosquito bites, but this is not certain.

The significant difference in antibody prevalence between males and females born before 1905 is puzzling. As far as is known, there has been no change in dress or custom since that time, so that it seems that the only explanation would be that during one of the later epidemics, for some unknown reason, females were attacked at a higher frequency than males.

Variation in antibody prevalence between climatic and geographic regions

It is apparent from Table 3 and Fig. 3 that there are marked differences in antibody prevalence between the regions of Fiji. The rates varied from as high as 29·5 % in Taveuni to as low as 9·65 % in the inland villages of Northern Vanua Levu. The low level of 8·6 % in Viti Levu is abnormal since the sample tested was not a true cross-section of the population.

Taveuni has a high rainfall and a dense mosquito population similar to that found in Southern Vanua Levu. Thus these two regions could be expected to have had higher numbers of infections, while Northern Vanua Levu, which is drier and has a lower mosquito density, would be expected to have fewer cases. In both regions, the observed antibody prevalence fits the climatic conditions. The relatively lower infection rates in the Lau Islands and Kadavu could be due to isolation. Travel and other forms of direct contact with these islands was rather limited at the time of the known epidemics. One observation not shown in Table 2 was the significant difference between the antibody prevalence in people who lived on volcanic islands and that of people who lived on the raised coral islands of the Lau Group. Both island types are populated by Fijians, those on the coral islands living mainly in fishing communities, while those on volcanic islands concentrate more on agriculture, and thus spend more time in the vicinity of coconut plantations. As expected from these observations, the prevalence of antibody on the coral islands was lower (9·2 %) than that found on the volcanic islands (15·2 %).

There was an interesting difference between the coastal and inland villages of Southern Vanua Levu, and the settlements and estates from the same region. Both coastal and inland villages had reasonably high infection rates, but the rates for the settlements were down to less than half that of the villages. It is known that the mosquito *Aë. polynesiensis* is found in the coastal areas and that further inland it is replaced by smaller numbers of *Aë. pseudoscutellaris*. Provided that there was no difference in the vector capability of the two species, one would expect a lower antibody prevalence in the inland villages, but such a difference was not observed. On the other hand, the settlements, being both coastal and inland, could have been expected, on purely geographical grounds, to have the same infection rates as the villages, but instead, they were considerably lower. It thus seems that the difference does not depend on the species of the vector. It seems more likely that the type of housing and the nature of the community are more important, the villages being compact groups of houses close to the bush, while the settlements usually

consist of isolated houses or 'barrack'-type dwellings in more open country. It is also probable that the chances of settlement dwellers becoming infected were considerably lower if the epidemic passed through the community rapidly.

Age differences and the size of previous epidemics

When the age distribution of the population tested during the whole of the survey period is compared with the age distribution of the total population of Fiji (Zwart, 1968), it can be seen that they are nearly identical. It therefore seems reasonable to draw certain conclusions from the serological status of the population as shown in Fig. 2.

The very low antibody prevalence in persons born since 1950 fits well with the fact that few cases of dengue have been notified during this period. Whether the antibody detected in these persons is due to past dengue infection or a low incidence of infection with a related virus, or merely a broad response to a variety of other infections, is not clear, but the fact that the titres in the sera from this age-group are generally lower than in other age-groups would indicate that the antibody may not be due to dengue virus infection.

After the 1943 epidemic in Fiji cases are known to have occurred for several years, and this would explain the antibody detected in person born between 1945 and 1950.

It is possible from the data presented in Fig. 2 to make an estimate of the number of susceptible persons infected during each epidemic. When the data given in the figure are plotted on semi-log paper, three sharp rises in antibody prevalence can be seen. One of these corresponds to the 1943 epidemic and from the graph it can be calculated that 23 % of susceptible persons became infected during the 5-year period embracing the epidemic. The next corresponds to the 1930 epidemic, in which 14 % of the susceptibles were infected during the 5-year period. The last corresponds to an epidemic which must have occurred around 1905 in which 17 % of susceptible persons were infected over a 5-year-period. There is no historical record of such an epidemic. These are figures for the whole population, but if the age distributions of antibody prevalence in different districts shown in Fig. 3 are studied, it can be seen that in some of these regions the infection rate could have been as high as 40 %. For the 20-year-period from 1907 to 1927, during which there were no known epidemics, the infection rate was 1·3–1·4 % of susceptibles per year.

Fig. 3 gives some indication of the history of dengue in different regions of Fiji. For example, Taveuni and Southern Vanua Levu present similar patterns, but Taveuni had much larger epidemics, as one would expect from the wet climate. On the other hand, Lau seems to have experienced the same epidemics on a smaller scale than Southern Vanua Levu. In contrast to these areas, Northern Vanua Levu inland and coastal villages seem to have missed the 1930 epidemic. Another interesting observation is that in Lau, Taveuni and Southern Vanua Levu there was no increase in antibody prevalence between the years 1910 and 1929. This would suggest that dengue was not endemic in these areas during this period.

Persons under the age of 20 years now represent approximately 57 % of the

total population of Fiji and over 99 % of these people have no immunity to dengue virus. Even in the total population the prevalence of non-immunes is 86 %. This must make Fiji a high risk area for a further epidemic of dengue in the future. In the absence of a satisfactory dengue vaccine, it would seem that very vigorous mosquito control programmes would be the only way to minimize this risk. Even if such programmes could be and were carried out, it is difficult to see how Fiji could expect to remain free of dengue when communications between Fiji and areas of known dengue endemicity have become so efficient. Added to the risk of the reintroduction of the same dengue virus type which caused the existing antibodies is the very real risk that a different type of dengue virus will appear there. In an explosive epidemic which occurred in Tahiti in 1964–5 and which was caused by dengue type 3, it was found that many people who had been infected during the 1940s with type 1 became reinfected with the new type (Laigret, Rosen & Scholammer, 1967). This would mean that if a new type of dengue was introduced to Fiji, considerably more than 86 % of the population would probably be susceptible.

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Mosquito-borne infections in Fiji

III. Filariasis in northern Fiji: epidemiological evidence regarding the mechanisms of pathogenesis

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SUMMARY

During a filariasis survey conducted in northern Fiji in 1968–9 examinations were made for microfilaraemia, enlarged lymph nodes and elephantiasis. Analysis of the microfilarial densities at different ages and the number of anatomical sites showing lymph gland enlargement or elephantiasis have been used to provide evidence on the clustering of infections and pathogenesis.

Although there is no evidence of clustering of risk of infection, there is evidence favouring the clustering of adult filariae in individuals. Nevertheless the number of sites of lymph node enlargement do not correspond with this finding and statistical evidence suggests that lymph-node enlargement is not necessarily associated with the near presence in the body of adult filariae, whether dead or alive.

Males of Indian ethnic origin showed a higher prevalence of elephantiasis than males of Fijian ethnic origin, but women of either ethnic race showed prevalences lower than those of men.

The onset of elephantiasis at a site does not directly reflect the number of infections sustained in the local area, but it appears that filariasis first induces for a limited period a proneness to elephantiasis. During this period a random and discrete event may induce the onset of elephantiasis. The nature of the event is unknown, but it probably is not trauma.

INTRODUCTION

Acute lymphangitis and lymphadenitis, localized inflammation with or without abscess formation, were shown definitely to be associated with infection by the filarial nematode *Wuchereria bancrofti* (Lewis, 1875; Manson, 1875). In endemic areas it was found that enlarged lymph nodes often contained living or dead adult filariae (Bahr, 1912; O'Connor, 1932). Moreover, although there was at one time argument whether elephantiasis was in fact caused by *W. bancrofti* it is now generally accepted that these filariae play an important part in the induction of this affliction. Nevertheless there are still differences of opinion as to whether enlarged

lymph nodes and elephantiasis at a particular site are due to the local presence of adult filariae or are reactions resulting from the presence of filariae anywhere in the body (Lane, 1937; Wartman, 1947; Manson-Bahr, 1952; Nelson, 1966; Beaver, 1970). Likewise the pathogenesis of elephantiasis, including the influence of extraneous factors such as trauma (Selwyn-Clarke, 1961; Jordan, 1954), still awaits further clarification.

Recently there have been available laboratory models employing experimental animals representing the human picture (Schacher & Sahyoun, 1967). In order to compare these models with the human disease it will be necessary to a certain degree to compare what may be termed the dynamics of the pathogenesis; for example, the intensity, duration and internal localization of infections. With regard to human infections, a great deal of the required information could have been obtained by longitudinal surveys of many years duration, but for reasons of practicability they were not done in endemic areas. Now that drug treatment is available such longitudinal surveys are for ethical reasons virtually impossible. Hence information must be sought from the analysis of surveys made over a short period of time, but including individuals of different ages.

The more obvious factors likely to influence the development of lesions of filariasis are the duration of exposure (age), risk of acquiring an infection, clustering of infections within the individual, and rates of recovery. The incidence of extraneous factors such as trauma may also be considered, if they are known or indicated. The data from which the factors can be evaluated must be reliable and assessable in a numerical form. The presence of microfilaraemia, the presence of lesions, enlarged lymph nodes and elephantiasis, and the number of sites of the lesions on individuals are obtainable with relative ease and good accuracy. Figures of the prevalence among individuals of the memory of episodes of fever or lymphangitis have been used in the past, but we consider that figures based on the memory of self-diagnosed clinical conditions are open to too many interpretations for meaningful discussion.

We have analysed data on filarial lesions obtained during a filariasis survey in northern Fiji, 1968–9 (Mataika, Dando, Spears & Macnamara, 1971), in order to determine which different theories of pathogenesis might fit the observed epidemiological patterns of lesions.

METHODS

A survey of filariasis in northern Fiji on the islands of Vanua Levu, Taveuni and Koro was conducted in 1968 and 1969. The survey has been described in detail by Mataika *et al.* (1971) in regard to the examination for microfilaraemia of population samples. All persons examined for microfilaraemia also received the clinical examination described below. Additional to these persons, a high, but not specifically selected, proportion of the population of Taveuni and southern Vanua Levu were examined for enlarged lymph nodes. An attempt was made to examine and enumerate all individuals in Taveuni and southern Vanua Levu who were suffering from elephantiasis.

Clinical examination

On both sides of the body the epitrochlear, axillary, inguinal and popliteal lymph nodes were examined for enlargement. The criterion for enlargement was the presence at the site of examination of one or more distinctly palpable glands being at least 1·5 times the normal size. Small shotty glands were not considered enlarged.

The limbs, breasts and genitalia were examined for elephantiasis, which was accepted as being present if there were signs and symptoms of sustained oedematous swelling with chronic skin changes.

RESULTS

Prevalence of microfilaraemia

These results have been presented already by Mataika *et al.* (1971).

Intensity of microfilaraemia (microfilarial density)

The results of surveys among Fijians in Taveuni and southern Vanua Levu are shown in Table 1.

Table 1. *Microfilariae in 20 mm.*^{3} *blood (Mf. D.) of Fijian male carriers in two areas of northern Fiji*

Age (years)		Taveuni & Koro	Southern Vanua Levu Coastal villages
5–9	No. examined	11	10
	Mf. D.*	9	6
	S.D. L†	0·63	—
10–14	No. examined	12	5
	Mf. D.	10	5
	S.D. L	0·64	—
15–19	No. examined	10	9
	Mf. D.	14	3
	S.D. L	0·47	—
20–29	No. examined	34	27
	Mf. D.	22	9
	S.D. L	0·53	0·68
30–39	No. examined	23	41
	Mf. D.	15	25
	S.D. L	0·68	0·60
40–49	No. examined	12	26
	Mf. D.	36	12
	S.D. L	0·55	0·49
50–59	No. examined	18	14
	Mf. D.	8	17
	S.D. L	0·45	0·80
60+	No. examined	11	11
	Mf. D.	9	7
	S.D. L	0·51	0·77

* Mf. D. = Geometric mean number.
† S.D. L = standard deviation of $\log_{10}$ of individual Mf. D.

Among those harbouring microfilariae in every group or subgroup in which sufficient data were collected, it was observed that the distribution among individuals of the number of microfilariae per 20 mm³ of their blood approximated a log.-normal distribution. For the various groups the geometric mean number of microfilariae per 20 mm.³ blood has been calculated. The standard deviation has been calculated from the transformed figures:

$$\text{S.D. } L = \text{standard deviation of } y,$$

where

$$y = \log_{10} \text{ microfilariae per 20 mm.}^3 \text{ blood.}$$

Enlarged lymph nodes

For individuals living in different areas and for different age-groups within these communities the prevalence of enlarged lymph nodes and the prevalence of microfilaraemia showed a moderate correlation among males, but among females the correlation was low. Hence the main analysis of prevalence of enlarged lymph nodes has been made in regard to males. The results are presented in Table 2.

The distribution of the number of anatomical sites showing lymph-node enlargement among Fijian males from three different areas and among 67 Fijian males

Table 2. *Prevalence of enlarged lymph nodes at one or more sites among Fijian males in three areas*

		Area		
			Vanua Levu	
Age (years)	Fijian Males*	Taveuni Koro	Southern coastal	Northern coastal
0–4	Ex	416	277	76
	%+	7·7	1·1	0·0
5–9	Ex	445	275	79
	%+	22·8	5·1	6·3
10–14	Ex	421	220	64
	%+	27·1	31·2	6·3
15–19	Ex	210	131	37
	%+	43·8	19·8	27·0
20–29	Ex	297	167	37
	%+	45·1	14·4	13·5
30–39	Ex	238	187	45
	%+	50·0	29·4	22·2
40–49	Ex	185	112	31
	%+	60·5	40·2	12·9
50–59	Ex	143	89	34
	%+	58·3	56·2	20·0
60+	Ex	84	91	16
	%+	71·4	54·9	37·5
All ages	Ex	2442	1549	419
	%+	34·7	19·1	12·2

* Ex = number examined. %+ = percentage with enlarged nodes.

Table 3. *Distribution and mean number of sites of lymph node enlargement among Fijian males*

Area	Description of population	Age (years)	No. exam.	% with enlargement at no. of sites equal to or more than:					Mean no. sites in cases with enlargement at one or more sites
				1	2	3	4	5	
Taveuni and Koro	Without Mf	⩾ 30	90	61	41	13	10	0	2·06
	With Mf	⩾ 30	62	32	25	6	3	0	2·06
	Unselected	⩾ 30	650	58·6	38·0	13·3	5·9	0·5	1·98
	Unselected	All ages	2442	34·6	19·0	5·3	2·0	0·1	1·77
Southern Vanua Levu coastal	Without Mf	⩾ 30	114	28	15	2	0	0	2·06
	With Mf	⩾ 30	89	37	9	1	0	0	1·27
	Unselected	⩾ 30	479	41·6	23·9	7·4	3·3	0·4	1·84
	Unselected	All ages	1549	19·1	9·9	2·4	1·0	0·1	1·71
All areas (limited survey)	Elephantiasis patients	⩾ 30	67	88·0	68·6	31·3	11·9	1·5	2·29

with elephantiasis observed in Taveuni and southern Vanua Levu are given in Table 3.

Also shown are data on individuals who were examined both for enlarged lymph glands and microfilaraemia. An analysis of data pertaining to males of all age-groups coming from Taveuni and southern Vanua Levu showed that in order to make the distribution of the number (x) of anatomical sites showing lymph-node enlargement one that is nearly normal, transformed figures (z) should be used where $z = x^{-0.867}$. The exponential is sufficiently near to 1·0 to indicate that the distribution is virtually arithmetic-normal (Taylor, 1961).

Elephantiasis

The population at the time of the survey has been estimated as that of the 1966 census (Zwart, 1968) with an additional natural increase of 5·5 %. From these estimates and the observed number of cases of elephantiasis, prevalences have been calculated for various population groups. Since some cases of elephantiasis may have been overlooked the prevalences given may be considered as the lowest estimates.

Table 4. *Minimum estimated prevalence of elephantiasis among the population of Taveuni, Koro and southern Vanua Levu*

Age (years)	Fijians				Indians			
	Males		Females		Males		Females	
	No.	%	No	%	No.	%	No.	%
0–9	0	0	0	0	0	0	0	0
10–14	1	0·1	1	0·1	0	0	0	0
15–19	1	0·1	2	0·2	0	0	0	0
20–29	5	0·3	5	0·2	2	0·6	4	1·2
30–39	20	1·2	20	1·2	6	2·8	6	3·2
40–49	29	2·5	20	1·9	10	6·8	2	1·9
50–59	54	6·8	33	4·5	9	11·3	1	1·5
60+	42	6·7	20	3·3	7	9·0	0	0
All ages	152	1·08	101	0·73	34	1·59	13	0·65

No. = number observed. % = percentage of population.

In Table 4 are shown for Fijians and Indians in Taveuni and southern Vanua Levu prevalences of elephantiasis according to age; and in Table 5 are shown for the same groups the distribution of the number of anatomical sites with elephantiasis among patients. At the foot of the table are given the percentages which are cumulative as the number of sites decreases. The percentage prevalence is reduced by a factor of about 2·6 for each additional site affected in males, and by a factor of about 3·2 in females.

Among Fijian males afflicted with elephantiasis at only one anatomical site the ratio of those with lesions in the right arm to those with lesions in the left arm was 1·2:1; and as regards legs the numbers were nearly equal; yet the number of

Table 5. *Distribution of number of anatomical sites affected with elephantiasis in Fijians (ref. also Table 4)*

Age (years)	Males: % with lesions at specified no. of sites 1	2	3	4	Males: Mean no. sites	Females: % with lesions at specified no. of sites 1	2	3	4	Females: Mean no. sites
0–9	0	0	0	0	—	0	0	0	0	—
10–14	(100)	0	0	0	1·00	(100)	0	0	0	1·00
15–19	(100)	0	0	0	1·00	(100)	0	0	0	1·00
20–29	(80)	0	(20)	0	1·40	(180)	0	(20)	0	1·40
30–39	85	10	5	0	1·25	90	10	0	0	1·10
40–49	69	17	14	0	1·45	85	15	0	0	1·25
50–59	57	26	11	6	1·65	61	27	6	6	1·58
60+	43	38	5	14	1·90	50	30	15	5	1·75
All ages	61	24	9	6	1·61	71	20	6	3	1·41
Cumulative % (all ages)	100	39	15	6	—	100	29	9	3	—

patients giving a history of severe trauma to the right arm were at least seven times greater than those giving histories related to the left arm. In regard to legs the ratio was 2 to 1.

DISCUSSION

In this discussion we shall consider the pathogenesis of the filarial infection uninfluenced by specific therapeutic drugs.

Ethnic and genetic susceptibility or tolerance to infection

The high prevalence of elephantiasis among Indian men suggests a true ethnic difference. To some extent the higher prevalence among Indian than among Fijian men under similar conditions of exposure to infection may be exaggerated by the slighter build of Indians, making minimal signs of elephantiasis more conspicuous and easily recognizable.

Sex differences in prevalences of enlarged glands and elephantiasis parallel those of differences in prevalence of microfilaraemia. Since it has been indicated by Mataika *et al.* (1971) that, among Fijians at least, the sex difference regarding microfilaraemia may be largely determined by recovery rate and not exposure risk, it is suggested that the same factor may influence the other manifestations.

Load of infection

Mataika *et al.* (1971), by observing age-prevalences of microfilaraemia in the population, indicated the risk of acquiring microfilaraemia. In a limited search, moreover, they were unable to show evidence of an appreciable clustering of risk of acquiring microfilaraemia. A clear distinction must be made, however, between a clustering of risk of infection and a clustering of the numbers of reproductively active filariae which survive in an individual and manifest their presence by liberating microfilariae. Hairston & Jachowski (1968) have indicated that such clustering within individuals occurs among the people of Samoa; and the rise of up to six times in the microfilarial densities between those of the younger and those of the older age-groups in our observations could be attributed to a similar clustering (Table 1). It was with a view towards elucidating the problem of clustering that the observations were made on the number of sites of lymph node enlargement (Table 3). The plan of the survey was based on the hypothesis that lymph-node enlargement at a site was due to the local presence of one or more adult filariae. The finding that the statistical distribution of the number of sites with enlargement is arithmetic-normal, but not approaching a Poissonian distribution if individuals without any enlargements are considered, indicates that enlarged nodes are not caused by the near presence of adult filariae, and cannot be used to evaluate clustering. The evidence for clustering is still based primarily on the work of Hairston & Jachowski with the supporting evidence of microfilarial densities. Clustering not being related to risk of infection could be due to host–parasite relationship, or interparasite effects producing either a synchronism of activity or a regression, if the clustering became too high.

Associated factors

There is unlikely to be much variation in the virulence of the parasite in the relatively uniform epidemiological situation in Fiji. The immune response is still too little understood to be discussed further.

The enlargement of lymph nodes is certainly associated with filarial infection, although in Fiji as elsewhere there are many other causes (O'Connor, 1932; Jordan, 1955). From our evidence it appears that in individuals with filarial infection there is a factor which almost regardless of the number of adult filariae induces enlargement of lymph nodes, at a random number of sites, but with a mean of one to two sites. Once enlargement has been induced regression is slow, so that subsequent infections may increase the mean number of sites. The presence of microfilariae in the blood and escaping into the tissue spaces could be a factor.

Although there are causes of elephantiasis other than filariasis, it may be assumed from the work of Bahr (1912) that in Fiji filariasis is the main predisposing factor. If elephantiasis resulted merely from an accumulation over the years of filarial infections then the incidence rate (the number of new cases arising per unit time) could be expected to show a gradual increase with age until finally it became equal to the incidence rate of filarial infection. Nevertheless we found that after the initial rapid rise in the age-prevalence percentage the percentage reaches a plateau or may fall (Table 4). Such a finding is not unique to Fiji (Hayashi, 1962; Wilson, 1961). Since recovery from elephantiasis is rare, we might account for the plateau of prevalence in the older age-groups by postulating an increased mortality among patients. Such a postulated mortality rate, however, would be unrealistically high. Hence a combination of a slightly increased mortality among cases and a rapidly diminishing incidence rate after the age of peak incidence is indicated. Such a situation could occur if susceptibility to the development of elephantiasis rose during the 35- to 50-year age period and was subsequently lost, not to be reacquired.

Elephantiasis is associated with enlarged lymph nodes in over 80% of cases, a figure significantly higher than that of the remainder of the population of similar age (Table 3). The mean number of sites of lymph-node enlargement in elephantiasis patients is slightly higher than that of unaffected individuals, but, considering the unhealthy state of the elephantiasis skin, it is not greatly higher. The numerical distribution of the number of sites showing elephantiasis is different from the distribution of lymph-node enlargement. There is a constant proportion in the reduction of prevalence for each additional site affected. This evidence indicates that among individuals susceptible or prone to elephantiasis the lesion itself is induced at a site by a randomly discrete event. That trauma is not the discrete event (Selwyn-Clarke, 1961) is indicated by the comparative divisions of prevalence between right and left sides for histories of trauma and for lesions of elephantiasis. Sepsis could be a cause.

Although we have not been able on epidemiological evidence to show the exact cause of the lesions in filariasis, we have been able to indicate the patterns of pathology into which theories should fit and to point out where existing theories may be inadequate.

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The growth and persistence of foot-and-mouth disease virus in the bovine mammary gland

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SUMMARY

In animals exposed to foot-and-mouth disease virus by indirect contact, virus was recovered from the blood, milk, pharynx, vagina and rectum for variable periods of time before clinical disease was apparent. Virus instilled into the mammary gland multiplied rapidly and virus concentrations greater than 10^7 p.f.u./ml. were recorded within 8–32 hr., depending on the virus strain and dose inoculated. Virus multiplication was accompanied by clinical signs of mastitis but the classical signs of foot-and-mouth disease did not appear for 52–117 hr. Dissemination of virus from the mammary gland occurred within 4–24 hr. and in some animals samples taken from the pharynx, mouth, nose and vagina contained virus for periods up to 97 hr. before the appearance of vesicular lesions. Virus production in the udder declined with the appearance of virus neutralizing activity in the blood and the milk but persisted in some animals for periods of 3–7 weeks. The ability of foot-and-mouth disease virus to persist in mammary tissue was confirmed by the demonstration of virus multiplication in the udders of immune animals.

INTRODUCTION

The presence of foot-and-mouth disease virus (FMDV) in the milk of some cattle incubating or exhibiting signs of the disease has been demonstrated or inferred on many occasions since the original observations of Lebailly (1920), and those concerned with the control of the disease recognize the importance of milk in the dissemination of virus both within and beyond the immediate locality of an infected farm. Examples of the spread of disease associated with infected milk have been recorded in the Report of the Departmental Committee on Foot-and-Mouth Disease 1952–54 and by Dawson (1970).

During the series of outbreaks of FMD in England and Wales in 1967 and 1968, virus was recovered from milk which had been collected from farms before the disease was diagnosed; virus concentrations of 10^4 and $10^{5.5}$ ID50/ml. (mouse) were measured in samples taken from a bulk milk tanker and from a churn respectively (Hedger & Dawson, 1970). Experimental infections of dairy cattle with the strain of virus concerned confirmed that high concentrations of virus could be found in the milk of some animals for several days before the appearance of clinical disease (Burrows, 1968).

This paper records the results of experiments which show that the bovine mammary gland is an important site of FMDV multiplication and possibly of the persistence of some strains of FMDV.

MATERIALS AND METHODS

Virus strains

1. O_1-BFS 1860: British field sample collected from cattle in the Wrexham area, 1 November 1967, and used after one passage in cattle at Pirbright.

2. A_{22}-Iraq 24/64: World Reference Laboratory sample used after two passages in cattle at Pirbright.

Cattle

A heterogeneous collection of commercial dairy cattle was used (ten Ayrshire, four Friesian and two Jersey). They varied in age from 3 to 8 years and, apart from one newly calved animal, were in middle to late lactation. Only two animals (supplied by the Institute for Research in Animal Diseases, Compton, through the courtesy of Professor W. M. Henderson) had a known history and could be regarded as mastitis-free at the beginning of the experiment.

The cattle were milked by hand twice daily during the early stages of experiment. During the later stages they were milked by machine once or twice a day, according to the requirements of the individual animal. Normal dairy hygiene was practised in respect of udder washing and disinfection between animals.

Infection, sampling and examination procedures

Animals exposed to infected 'donor' cattle, sheep and pigs

The animal accommodation consisted of 12 loose boxes opening on three sides of a central area which had stalls for ten cattle. The 'donor' animals were placed in three boxes and the 'recipient' dairy cattle were placed in the central area. The two groups were separated by at least 10 metres and were handled by different attendants.

Air movement in the animal area was from intake points in each loose box to extraction points above the stalls in the central area. The 'donor' animals were infected by the intradermal inoculation of the tongue (one steer), the coronary band (four sheep) and the bulb of the heel (four pigs). Under these conditions, trace amounts of virus have since been detected in the communal air of the shed within 24 hr. and considerable amounts within 48 hr. (Sellers & Parker, 1969).

The 'recipient' animals were examined each morning for clinical signs of disease and samples of blood and pooled quarter milk were collected. Swabs were taken from the vaginal and rectal cavities, and oesophageal/pharyngeal samples were collected as described by Burrows (1966). Rectal temperatures were recorded during the early morning and late afternoon.

Animals infected by udder inoculation

Two ml. of a virus suspension were introduced into the milk sinus of the right hind quarter, using a teat cannula. Oesophageal/pharyngeal samples and nasal, conjunctival, vaginal and salival swabs were taken each morning and afternoon. Clinical examinations were carried out, rectal temperatures recorded, and blood and individual quarter milk samples were collected every 4 hr. until clinical disease was apparent.

Some animals were retained on experiment for up to 12 weeks. During this period samples were collected at irregular intervals and examined for antibody content and for evidence of virus persistence.

Detection and assay of virus and neutralizing antibody

Oesophageal/pharyngeal specimens, blood and milk collected during the day were tested for infectivity within 1–3 hr. of collection. Samples taken during the night were stored at 4° C and examined the following morning. Blood samples were allowed to clot and only the serum screened for infectivity. Swabs were stored at −20° C for 1–3 weeks before being processed in 3 ml. of diluent immediately before examination.

The majority of samples were examined in parallel in two culture systems. Trace amounts of virus were detected in primary monolayers of calf thyroid (BTY) cells in roller tubes (Snowdon, 1966). Larger amounts of virus were assayed by counts of plaque forming units (p.f.u.) in monolayers of the pig kidney cell line IB-RS-2 (Instituto Biologico-Renal Swine-2; de Castro, 1964). Because high concentrations of milk damaged the tissue cultures, it was found necessary to remove the inoculum and rinse the monolayer with diluent before adding the nutrient fluid or agar overlay.

The identity of virus strains was monitored periodically by complement fixation and neutralization tests, using type specific sera. Serum neutralizing antibody titres were determined by the cell metabolic inhibition test (Martin & Chapman, 1961). Comparisons of milk and serum neutralizing antibody content were carried out by means of a plaque reduction test on IB-RS-2 monolayers, using a constant virus/variable serum or milk dilution procedure similar to that described by Federer, Burrows & Brooksby (1967).

RESULTS

Cattle exposed to an indirect contact infection

Experiments were carried out with both the O_1 and A_{22} strains of virus. In each experiment the four dairy cattle formed part of a large group of recipient cattle, sheep and pigs (Burrows, 1968).

Clinical observations

The period of time which elapsed between infection of the donor animals and the appearance of disease in the recipient dairy cattle ranged from 5 to 8 days.

Above average rectal temperatures were recorded for four of the eight cattle during the 24 hr. preceding the appearance of vesicles and for six animals at the time when lesions were first seen. The first crop of vesicles was detected in the interdigital regions and the bulbs of the heels of one or more feet (six animals); four of these also had vesicles on the dental pad or lips. One animal had lesions on the dental pad only and one animal on one teat only. During the following 24–72 hr. further crops of vesicles appeared at the majority of susceptible sites in the mouth, on the feet and on the teats of all animals. A mucoid nasal discharge was seen in some animals and this resulted in some degree of excoriation of the outer nares and muzzle. Milk production dropped by 30–50 %, commencing on the day that lesions were first seen, and the milk of five of the cattle contained obvious clots. Bacteriological and cytological examinations of the milk were carried out in the A_{22} experiment. No significant changes in the bacterial flora took place and the cellular content of the milk did not increase more than three-fold. Milk pH values remained within the limits of 6·5 to 6·9.

Virological findings

The amounts of virus recovered from samples taken before the appearance of clinical disease are listed in Tables 1 and 2. Virus was recovered from the pharynx, blood and milk of 7 of the eight cattle, from the vagina (four cattle) and from the

Table 1. *Virus content of samples taken before the appearance of clinical lesions* (*indirect contact infection*, O_1)

Animal no.	Sample origin	Day of experiment 2	3	4	5	6	7	8
GI 27	Serum	—	—	—	4·0*	Lesions		
	Pharynx	—	—	2·5	> 4·5			
	Milk	—	—	—	3·3			
	Vagina	—	—	—	2·9			
	Rectum	—	—	—	1·6			
GI 28	Serum	—	—	—	—	1·7	2·1	Lesions
	Pharynx	—	2·8	2·5	3·1	4·0	5·5	
	Milk	—	—	1·4	3·5	4·6	4·5	
	Vagina	—	—	—	—	—	—	
	Rectum	—	—	—	—	—	—	
GI 29	Serum	—	—	1·1	4·1	Lesions		
	Pharynx	—	—	3·5	> 4·5			
	Milk	—	—	1·0	5·2			
	Vagina	—	—	—	> 3·3			
	Rectum	—	—	—	1·0			
GI 30	Serum	—	—	1·1	1·0	Lesions		
	Pharynx	—	—	2·5	3·5			
	Milk	—	—	1·3	2·4			
	Vagina	—	—	—	> 3·3			
	Rectum	—	—	—	—			

* Log_{10} p.f.u./ml. (serum, milk) or per sample (oesophageal/pharyngeal samples, vaginal/rectal swabs).

— No virus recovered.

rectum (two cattle) for variable periods of time before disease was apparent. In the majority of animals, virus was first detected in those samples taken from the pharyngeal area. The geometric mean concentrations of virus ($\log_{10}$ p.f.u./sample) in the positive pharyngeal samples collected 5, 4, 3, 2 and 1 day before signs of disease were 1·0, 2·1, 3·5, 3·5 and $\geqslant$ 4·2. Although the presence and concentrations of pharyngeal virus during the pre-clinical stages of disease were similar in the two experimental groups, the occurrence and concentration of virus in other samples differed. In the A_{22} experiment virus was detected in only three samples

Table 2. *Virus content of samples taken before the appearance of clinical lesions (indirect contact infection,* A_{22}*)*

Animal no.	Sample origin	Day of experiment 2	3	4	5	6	7	8
GQ 20	Serum	—	—	—	—	> 2·6*		
	Pharynx	—	T	2·9	2·7	> 3·8	Lesions	
	Milk	—	—	—	—	2·8		
GQ 21	Serum	—	—	—				
	Pharynx	—	—	—	Lesions			
	Milk	—	—	—				
GQ 22	Serum	—	—	—	1·8	—	—	
	Pharynx	—	T	3·3	4·5	> 3·8	4·2	Lesions
	Milk	—	—	—	—	—	1·2	
GQ 23	Serum	—	—	—	2·4			
	Pharynx	—	—	5·3	> 3·8	Lesions		
	Milk	—	—	—	1·7			

* $\log_{10}$ p.f.u. per ml. (serum, milk) or per sample (oesophageal/pharyngeal).
T = trace amounts of virus detected in BTY cultures only.
– = no virus recovered.
Only one vaginal swab contained virus (GQ 22 Day 7–$10^{2.6}$ p.f.u.).
No virus recovered from rectal swabs.

of serum (mean infectivity 2·2), three samples of milk (mean infectivity 1·9) and in one vaginal swab. In the O_1 experiment virus was found in seven samples of serum (mean infectivity 2·16) and nine samples of milk (mean infectivity 3·02), three vaginal swabs and two rectal swabs. This slight difference in the distribution of virus found in the two groups of milking cows before the appearance of disease was not seen in the larger group of steers which were included in each experiment.

During the first 4 days of clinical disease the majority of milk samples contained considerable amounts of virus (29/32 – mean infectivity 3·09, range 1·2–5·2). Neutralizing antibody appeared in the sera of the cattle on the 8th and 9th days of the experiment (5–7 days after virus was first detected in the pharynx) and this coincided with a reduction in both the frequency and the amounts of virus recovered from the milk. The O_1 cattle were not sampled after the 12th day of experiment but the A_{22} cattle continued to excrete virus in the milk up to the 19th day.

Cattle infected by udder inoculation

Clinical findings

Although there was some individual variation in the thermal response of the animals to infection, the general pattern was the same. Sixteen to 24 hr. after instillation of virus into the udder, the rectal temperature increased over a period of 4–8 hr. to a maximum of 104·2 to 106·8° F. and then returned to near normal levels. A second increase in temperature up to 103–106·2° F. occurred 4–28 hr. before the appearance of vesicular lesions.

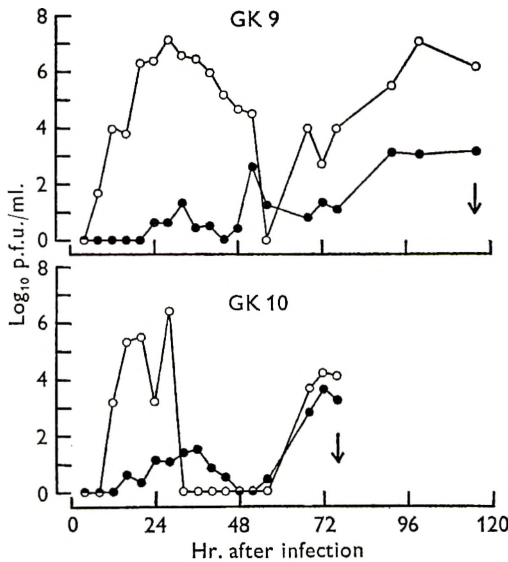

Fig. 1

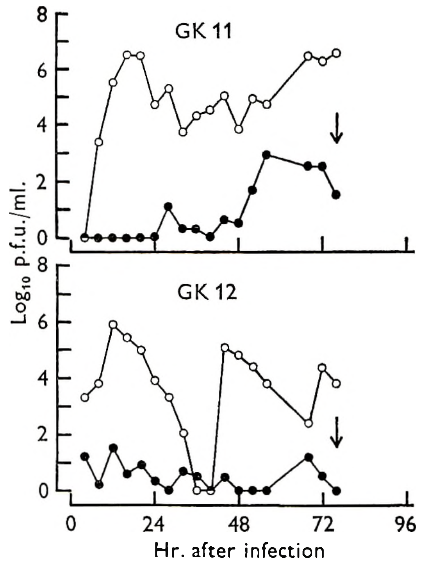

Fig. 2

Figs. 1–4. The occurrence and concentrations of virus in the milk before the appearance of generalized disease following udder inoculation. Fig. 1. O_1, 10^3. Fig. 2. O_1, 10^6. Fig. 3. A_{22}, 10^3. Fig. 4. A_{22}, 10^6. ○—○, Inoculated quarter; ●—●, geometric mean: non-inoculated quarters; ↓, appearance of generalized disease.

Fig. 2. For legend see Fig. 1.

All cows exhibited clinical signs of mastitis in the inoculated quarter within 28 hr. of infection. The affected quarter was slightly swollen, hot and tender and the milk was somewhat watery, slightly coloured, contained numerous clots and was considerably reduced in amount. Penicillin was given to reduce the possible activity of commensal bacteria and this resulted in an improvement to a less acute form of mastitis which progressed during the next 48 hr. to involve the non-inoculated quarters of the udder. The milk yield dropped to approximately 60 % of the pre-infection volume.

Signs of generalized disease developed between 76 and 117 hr. in the O_1 group (Figs. 1, 2) and between 52 and 88 hr. in the A_{22} animals (Figs. 3, 4). The first crops of vesicles appeared on the feet and these were followed during the next 48 hr. by vesicles in the mouth and on the teats.

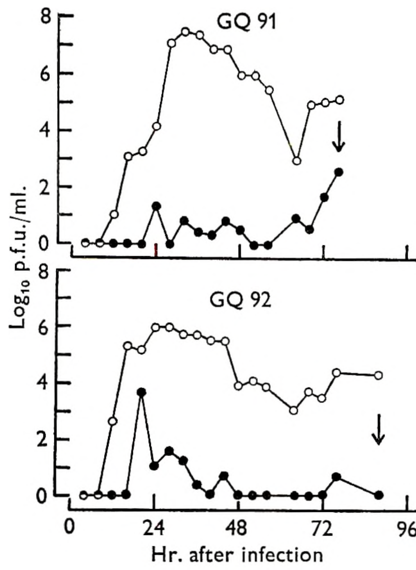

Fig. 3

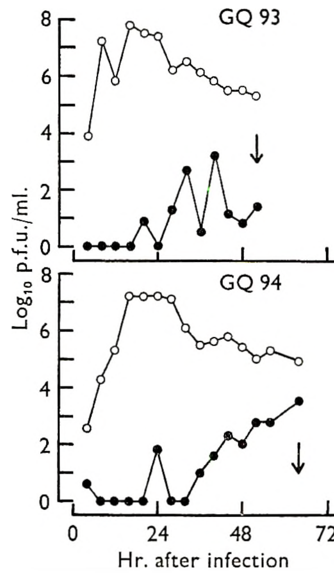

Fig. 4

Fig. 3. For legend see Fig. 1.

Fig. 4. For legend see Fig. 1.

Table 3. *Percentage frequency of virus recovery and mean infectivity of milk samples taken from different quarters prior to the development of generalized disease (udder inoculation)*

	O_1		A^{22}	
Quarter	Percentage frequency virus recovery	Mean infectivity of positive samples	Percentage frequency virus recovery	Mean infectivity of positive samples
Right hind	78	4·68*	93	5·36
Left hind	42	2·07	20	2·13
Right fore	37	2·18	34	2·46
Left fore	34	1·81	29	2·71

* Log_{10} p.f.u./ml.

Virological findings

The occurrence and concentration of virus in the milk before the appearance of generalized disease are detailed in Figs. 1–4. In animals given a small dose of virus (10^3 p.f.u.), the first indication of virus multiplication in the udder was obtained at the 8th and 12th hr. and peak virus concentrations were reached between 24 and 36 hr. (Figs. 1, 3). In the animals given the larger dose (10^6 p.f.u.), virus was present in the samples taken at 4 hr. in three cows and peak virus titres occurred between 12 and 20 hr. (Figs. 2, 4). In three of the O_1 cattle a complete loss of infectivity for various periods of time was found in samples collected between 32 and 56 hr. after infection. This feature was not evident in the A_{22} cattle, although some decrease in the virus content of the milk was found after the 32nd hr. Virus was recovered sporadically and at relatively low titre from the non-inoculated quarters

from 16 to 24 hr. after infection, although in two animals virus was recovered in the samples taken at 4 hr. No differences in the response of the non-inoculated quarters was apparent in respect of the frequency of virus recovery or of mean virus content (Table 3).

Table 4. *Virus content of samples taken before the appearance of clinical lesions (udder inoculation,* O_1*)*

Animal no.	Sample origin	Hours after infection 4	20	28	44	52	68	76	117
GK 9	Serum	—	T	T	1·1*	1·6	2·1	> 2·7	Lesions
	Pharynx	—	T	1·0	2·6	2·3	2·0	3·7	
	Saliva	—	1·1	2·6	2·2	2·6	—	2·7	
	Nose	—	—	—	2·9	2·9	3·0	2·0	
	Vagina	—	—	—	—	1·0	2·5	2·3	
GK 10	Serum	—	T	T	1·9	> 2·7	2·4	Lesions	
	Pharynx	1·6	2·6	3·6	3·6	3·2	4·0		
	Saliva	—	—	2·0	1·3	2·2	2·7		
	Nose	—	—	1·0	3·2	2·7	2·7		
	Vagina	—	—	—	2·6	1·7	2·5		
GK 11	Serum	—	T	T	2·4	> 2·7	> 2·7	Lesions	
	Pharynx	—	2·4	2·4	3·2	3·2	> 4·0		
	Saliva	—	—	1·4	1·7	> 2·7	> 2·7		
	Nose	—	—	—	3·3	> 3·3	> 3·3		
	Vagina	—	2·3	2·2	2·5	2·5	2·5		
GK 12	Serum	—	—	T	0·7	1·0	0·4	Lesions	
	Pharynx	—	—	—	3·0	2·0	3·5		
	Saliva	—	—	—	—	—	—		
	Nose	—	0·3	1·7	2·4	2·5	2·8		
	Vagina	—	2·0	1·0	2·0	2·0	2·5		

* Log_{10} p.f.u./ml. or sample.
T = Trace amounts of virus detected in BTY cultures only.
— = No virus recovered.
Animals GK 9 and GK 10 each received 10^3 p.f.u. of virus O_1.
Animals GK 11 and GK 12 each received 10^6 p.f.u. of virus O_1.

A feature of these experiments was the rapid dissemination of virus from the inoculated quarter to other sites, both in the udder and the pharyngeal area. Tables 4 and 5 list the virus content of samples collected at various times after inoculation and before the appearance of generalized disease. In the O_1 cattle, virus was detected in the pharynx of one animal 4 hr. after infection, in the blood of the two high virus dose cattle at 8 hr. and in the low virus dose group at 12 and 20 hr. Virus was found in nasal and vaginal swabs and in saliva collected from the mouth from the 20th hour onwards in some animals. In general, the virus content of the samples increased with time (Table 4) and must represent multiplication of virus in the pharyngeal, oral, nasal and vaginal mucosae at a level below that required to produce gross vesiculation.

A similar but less marked pattern of virus recovery was obtained for the A_{22} cattle (Table 5), although virus was not recovered from vaginal or rectal swabs before the 88th hour, by which time all animals had developed generalized disease.

Virus was detected in the pharynx of one animal at 16 hr. and in the blood of the high virus dose animals at 16 and 20 hr., and in the low virus dose pair at 20 and 24 hr.

Table 5. *Virus content of samples collected before the appearance of clinical lesions (udder inoculation,* A_{22})

Animal no.	Sample origin	Hours after infection 16	24	40	48	64	72	
GQ 91	Serum	—	1·5*	3·9	3·9	3·6	3·8	Lesions 76 hr.
	Pharynx	—	—	T	2·6	2·5	2·7	
	Saliva	—	—	—	T	—	—	
GQ 92	Serum	—	1·0	—	—	2·0	1·0	Lesions 88 hr.
	Pharynx	—	—	—	—	—	T	
	Saliva	—	—	—	—	T	3·6	
GQ 93	Serum	0·8	3·3	3·1	3·5	Lesions 52 hr.		
	Pharynx	T	2·7	—	3·1			
	Saliva	—	1·0	2·4	3·4			
GQ 94	Serum	—	2·3	3·0	3·4	Lesions		
	Pharynx	—	—	T	3·4			
	Saliva	—	—	2·7	2·8			

* $\log_{10}$ p.f.u. per ml. or per sample.
T = Trace amounts of virus detected in BTY cultures only.
— = No virus recovered.
No virus detected in vaginal or rectal swabs. Animals GQ 91 and GQ 92 each received 10^3 p.f.u. of virus A_{22}. Animals GQ 93 and GQ 94 each received 10^6 p.f.u. of virus A_{22}.

The virus content of the milk during the clinical and convalescent stages of disease is detailed in summary form in Table 6 which lists the total number of quarter samples examined, the number of samples from which virus was recovered and the means and ranges of infectivities recorded. In general, the virus content of samples remained high ($\geqslant 3\cdot0$ $\log_{10}$ p.f.u./ml.) for the first 2 or 3 days of obvious disease and then, with the development of serum antibody, the frequency of recovery and the virus content of samples decreased. In the A_{22} experiment approximately 85 % of all milk samples collected between the 4th and 10th days contained virus, no virus was detected on the 11th and 16th days, but virus was recovered from three quarters of one animal on the 23rd day. In the O_1 experiment approximately 65 % of quarter samples collected between the 4th and 10th days, 40 % collected between the 11th and 20th days, 34 % collected between the 21st and 30th days, and 12 % collected between the 31st and 84th days contained virus. Virus was recovered intermittently from two animals for 30 days and from the other two cattle for 44 and 51 days, respectively.

The occurrence of virus in the milk of convalescent cattle was unexpected in view of the high neutralizing activity of the milk of these animals. The appearance and concentration of neutralizing antibody in the milk in relation to that of serum is tabulated in Table 7. Serum antibody was first detected on the 4th or 5th day after udder inoculation; trace amounts of neutralizing activity were detected in the milk on the 6th day and considerable amounts on the 7th day and this

Table 6. *Infectivity of milk during the clinical and convalescent stages of FMD initiated by udder inoculation*

Days after infection	Cow*	O_1 No. of samples positive	O_1 No. of samples tested	O_1 Geometric mean infectivity	O_1 range	A_{22} No. of samples positive	A_{22} No. of samples tested	A_{22} Geometric mean infectivity	A_{22} range
4–10	1	25	36	3·26†	0·7–7·2	38	40	3·49	1·0–6·5
	2	22		3·60	1·0–5·9	30		2·67	0·7–4·9
	3	21		3·74	1·5–6·5	38		3·77	1·2–6·6
	4	25		2·47	0·7–3·5	32		3·38	1·0–5·8
11–20	1	9	32	1·40	0·7–2·0	0	8		
	2	13		1·58	0·7–3·9	0			
	3	2		2·85	2·2–3·5	0		.	.
	4	26		1·94	0·7–3·2	0			
21–30	1	11	28	1·59	0·7–3·5	0	8		
	2	2		1·45	0·7–2·2	0			
	3	7		1·21	0·7–2·0	3		1·33	1·0–2·0
	4	19		1·94	1·0–2·7	0			
31–84	1	2	32	2·75	2·5–3·0	0	24		
	2	0		—	—	0			
	3	12		2·07	0·7–3·7	0		.	.
	4	1		2·2	—	0			

* O_1: 1. GK 9; 2. GK 10; 3. GK 11; 4. GK 12. A_{22}: 1. GQ 91; 2. GQ 92; 3. GQ 93; 4. GQ 94.

† Log_{10} p.f.u./ml.

coincided with a decrease in the virus content of the milk. From about the 9th day onwards the level of milk antibody parallelled that of the serum, being approximately 14-fold lower (range 9- to 22-fold) in the O_1 group of cattle and approximately 4-fold lower (range 2- to 11-fold) in the A_{22} cattle. No obvious differences in these relationships were measured in animals producing 2 litres or 10 litres of milk a day or in the milk from individual quarters of an animal.

Re-infection of convalescent cattle by udder inoculation

The response of convalescent cattle to re-infection by the instillation of virus into the udder was examined 12–15 weeks after their first experience of FMD. Details of the experiment are given in Table 8, which shows that despite considerable neutralizing activity of the milk (90 % neutralization of test virus at dilutions

Table 7. *Virus neutralizing activity of serum and milk following FMD initiated by udder inoculation*

Days after infection	O_1		A_{22}	
	Serum	Milk	Serum	Milk
3	⩽ 0·3*	⩽ 0·3	⩽ 0·3	⩽ 0·3
4	⩽ 0·3	⩽ 0·3	0·95	⩽ 0·3
5	1·85	⩽ 0·3	1·85	⩽ 0·3
6	3·15	0·95	2·90	0·70
7	3·75	2·65	3·90	2·10
8	4·35	3·20	—	—
9	—	—	3·75	3·40
14	4·35	3·30	—	—
16	4·40	3·30	4·00	2·95
21–30	4·15	3·00	3·60	3·35
31–40	4·30	2·95	3·80	2·95
41–50	4·30	—	4·05	3·25
51–60	4·00	—	3·30	3·10
61–70	—	2·90	3·80	3·10

* Log_{10} reciprocal of the initial serum or milk dilution which neutralized 90 % of test virus.
O_1, geometric means for cows GK 9 and GK 11.
A_{22}, geometric means for cows GQ 92 and GQ 94.

of 1/200 to 1/500) it was possible to demonstrate a virus growth cycle in the inoculated quarter over a period of at least 90 hr. Twenty-four to 42 hr. after re-infection, a transitory swelling of the inoculated quarter along with some thickening of the milk was noted. No virus was recovered from the blood or from the non-inoculated quarters during 7 days of observation. Three of the four cattle were carrying virus in the pharynx at the time of re-infection.

DISCUSSION

Afshar & Bannister (1970) have reviewed the literature concerning viral infections of the bovine mammary gland and have listed the viruses which have been reported to multiply in the udder. These include the viruses of human influenza (PR. 8), Newcastle disease, fowl plague, mumps, canine distemper, poliomyelitis,

Table 8. *Virus multiplication in the mammary gland of convalescent cattle re-infected by udder inoculation*

Animal no.	History	Pre-infection antibody		Virus dose A_{22} ($\log_{10}$ p.f.u.)	Virus content of milk from inoculated quarter. Hours after infection						
		Serum	Milk		−1	18	24	42	66	90	168
GQ 92	84 days after infection (udder inoculation A_{22})	3·5*	2·3	5·0	0	4·2†	4·5	0	1·4	2·7	0
GQ 94	84 days after infection (udder inoculation A_{22})	3·3	2·6	3·0	0	1·7	3·0	3·0	0	0	0
GQ 23	105 days after infection (indirect contact A_{22})	3·5	2·5	5·0	0	3·3	3·5	0	0	0	0
GQ 20	105 days after infection (indirect contact A_{22})	3·0	2·7	3·0	0	0	3·5	3·2	1·4	0	0

* $\log_{10}$ reciprocal of the serum or milk dilution which neutralized 90 % of the test virus (A_{22}).

† $\log_{10}$ p.f.u./ml.

vesicular stomatitis, foot-and-mouth disease, infectious bovine rhinotracheitis, pseudo-cowpox, African swine fever, a bovine enterovirus and vaccinia and para-influenza 3 viruses. FMDV was included on the evidence that instillation of the virus into the udder was followed by generalized disease (Thomas & Leclerc, 1961). Some of these viruses have been reported to multiply without causing obvious clinical abnormalities, whilst others have been shown to produce changes in the mammary gland and its secretions which ranged from a transitory increase in the cellular content of the milk to the more obvious physical changes associated with acute mastitis.

The mastitis which has been described in some cattle experiencing FMD has usually been attributed to the activity of commensal bacteria following the appearance of vesicular lesions on the teats and udder. In the present series of experiments the mastitis was almost certainly due to virus proliferation within the mammary gland, as it occurred before the appearance of detectable lesions on the skin of the mammary regions and was accompanied by considerable amounts of virus in the milk.

Although, in the indirect contact experiments, the behaviour in steers of the O_1 and the A_{22} viruses was not noticeably different, small variations in the distribution and concentrations of virus were evident in the dairy cattle. These differences were more marked in animals infected by udder inoculation. The A_{22} virus appeared to be the more virulent virus in the milking cow in that it grew more readily in the udder, produced great virus concentrations in the blood, produced clinical lesions more quickly and did not appear to persist in the udder to the same extent as did the O_1 virus. During the period 20–48 hr. after infection, all the milk samples from the inoculated quarters of the A_{22} cattle and 39 % of samples from the other quarters yielded virus (mean infectivity in $\log_{10}$ p.f.u./ml.–6·03 and 2·33, respectively). The comparable figures for the O_1 cattle were 78 % (mean infectivity 4·96) for the inoculated quarters and 32 % (mean infectivity 1·40) for the other quarters. The reason for the disappearance of virus from the milk of some of the O_1 cattle for varying periods of time 30 or more hours after udder inoculation is not understood. A retrospective examination of the sera of these animals showed a transitory increase in virus inhibitory activity (up to initial serum dilutions of 1/64 measured by the cell metabolic inhibition test) during this period but the nature of this inhibition was not investigated, nor were the milk samples examined for inhibitory activity. The virus neutralizing activity of the milk appeared soon after that of the serum and was associated with a decline in the infectivity of the milk, which was more marked in the A_{22} cattle than in the O_1 animals.

This difference in virus growth in the udder was reflected in the concentrations of virus which were present in the blood during the pre-clinical period. In the A_{22} cattle, blood virus levels of 3·0 $\log_{10}$ p.f.u./ml. were measured in two cattle 20 hr. after infection and the mean infectivity levels of samples taken every 4 hr. from three of the four cattle were 3·43, 3·16 and 2·71 for the period between 20 hr. after infection and the time of appearance of disease. Although virus was detected in the blood of two of the O_1 cattle 8 hr. after udder inoculation and in the blood of the other two animals at 12 and 20 hr. and in all subsequent samples, the concentra-

tions of virus remained low and did not exceed 1·0 $\log_{10}$ p.f.u./ml. during the first 40 hr. Despite this low level of viraemia, the O_1 virus succeeded in localizing and multiplying at other sites more readily than did the A_{22} virus. Virus was recovered from 65 of the 100 samples taken from the pharynx, the mouth, the nose and the vagina of the O_1 cattle before the development of disease, whereas only 18 of the 80 samples taken from the pharynx, the mouth, the vagina and the rectum of the A_{22} cattle contained virus.

The appearance of virus in the pharynx of 'recipient' animals 3 or 4 days after the inoculation of 'donor' animals is not now surprising, in the light of present knowledge. Sellers & Parker (1969) have now shown that the virus content of the communal air in this animal compound is greatest at the time of appearance of generalized disease and this usually occurs 40–72 hr. after inoculation. Whether this 'pharyngeal virus' in the recipient animal is the result of air sampling by the upper or by the lower respiratory tract has not yet been resolved but the results of sequential post-mortem studies of animals infected by either natural or simulated natural means have indicated that the pharynx is the primary site of virus localization and multiplication in the ruminant (R. Burrows, J. A. Mann, A. J. M. Garland & D. Goodridge – unpublished work). The susceptibility of the pharynx to haematogenous infection as well as to surface infection was confirmed in the cattle infected by udder inoculation. Virus was detected in the pharynx of one animal 4 hr. after udder infection and in four of the eight cattle within 24 hr. Although the possibility of the pharynx having been infected by either the oral or respiratory route cannot be completely excluded, this is most unlikely as the four animals were fastened by neck chains at least 10 metres apart and precautions were taken to limit aerosol formation during milking procedures.

Although primary infection of the mammary gland is unlikely to be a common occurrence in the pathogenesis of FMD, the results of experimental inoculation of the udder show that it is a highly susceptible organ which is capable of producing large amounts of virus. This susceptibility is of some significance; the mammary gland of the lactating cow is richly supplied with blood and so may well be one of the first organs to become infected from the transitory viraemia which follows primary multiplication of virus in the pharyngeal area. Once the mammary tissue is infected, virus could be expected to appear in the milk fairly quickly, whereas virus localizing in the epithelium of the mouth or feet might require 24–30 hr. to produce recognizable vesicles. The susceptibility of the mammary gland tissue to FMDV may also explain the ability of virus to persist in this region. The recovery of virus from the milk of the O_1 cattle for periods up to 51 days and the A_{22} cattle for 23 days, and the susceptibility of convalescent A_{22} cattle to re-infection by udder inoculation, indicate that these strains of FMDV continued to multiply in the mammary tissue of the immune animal. The persistence of FMDV in the pharyngeal region of cattle for long periods is well documented (van Bekkum, Frenkel, Frederiks & Frenkel, 1959; Sutmöller & Gaggero, 1965; Burrows, 1966) but until now no evidence for the possible persistence of FMDV in other regions has been obtained.

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