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Laboratory investigation of sewer swabs following the Aberdeen typhoid outbreak of 1964

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The sewer-swab technique was used by Moore (1948) to trace a *Salmonella paratyphi B* excreter and later to track down a *S. typhi* excreter (Moore, Perry & Chard, 1952). Other investigators, using Moore's methods, have successfully traced *S. typhi* carriers (Lendon & Mackenzie, 1951; Pilsworth, 1960).

A programme of sewer swabbing, based on Moore's method, was begun in Aberdeen during the closing stages of the typhoid outbreak in an attempt to locate possible undetected excreters of *S. typhi* among the population at risk. Although this was the prime purpose of the work, the survey developed into an investigation of the efficacy of techniques used. Some 2300 sewer swabs were examined between July 1964 and September 1966.

MATERIALS AND METHODS

Sewer swabs were prepared and collected as described by Moore (1948, 1950) and Moore *et al.* (1952). These, placed in sewers on Mondays and Tuesdays, were collected 7 days later.

Media

Throughout the investigation the following commercially available media were used with supplements as indicated: (*a*) Difco bismuth sulphite agar with added ferrous sulphate solution (B.S.A.) as recommended by Hobbs (1943); (*b*) oxoid S.S. agar with 1% saccharose added (S.S.S.A.); this medium was employed as it forms the basis of all our routine cultural work for enteric organisms, is easy to prepare and plates may be stored for several days without loss in efficiency; (*c*) oxoid mannitol selenite broth (M.S.B.) similar to the medium described by Hobbs & Allison (1945) for the isolation of *S. typhi*.

Cultural methods

On arrival in the laboratory, swabs were inspected and quarter-strength Ringer solution added to just immerse each swab. The content of each swab was then thoroughly expressed into the added fluid using a stout sterile glass rod.

Method 1

Serial tenfold dilutions of the swab fluid were made in quarter-strength Ringer solution from 1/10 to 1/10,000. Centrifuged deposit of 5 ml. of swab fluid was also prepared.

S.S.S.A. and B.S.A. plates were seeded with 1 drop each of swab fluid and its dilutions, and with 1-, 2- and 3-drop inocula of the centrifuged deposit. Inocula were spread in parallel lines with an angled wire (1 cm. spreading edge) without return to the original inoculum and without flaming the wire at any stage of the spreading. To the swab with its free fluid and to each of the dilutions, equal volumes of double strength M.S.B. were added; the mixtures were incubated for 18–24 hr. at 37° C. and then subcultured to S.S.S.A. and B.S.A. All S.S.S.A. plates were examined after 24 hr. and B.S.A. plates after 24 and 48 hr. at 37° C. Thus, a total of 26 plate cultures was examined for each swab.

Non-lactose-saccharose fermenting colonies from S.S.S.A. and black colonies from B.S.A. were transferred to Christensen's urea medium. Urease negative organisms were tested in glucose, mannitol and dulcitol broths (Kauffman, 1950) and spread on MacConkey agar and nutrient agar slopes. Organisms giving suggestive fermentation reactions were finally identified by slide agglutination with appropriate antisera. An attempt was made to select up to ten colonies from each plate culture, so that a total of 260 colonies might be examined from each swab.

Method 2

Four plates of B.S.A. were inoculated in series with 1 drop of expressed swab fluid and 1 drop each of 1/10, 1/100 and 1/1000 dilutions of the fluid in sterile heart infusion broth (Difco). Three tubes of 4 ml. single-strength M.S.B. were inoculated with 1, 2 and 3 drops of undiluted swab fluid, while a further 4 ml. of the fluid were added to an equal volume of double-strength M.S.B. An equal volume of double-strength M.S.B. was added to the swab and its remaining fluid. M.S. broths, after incubation for 18–24 hr., were subcultured to S.S.S.A. only. These procedures gave a total of nine plates per swab. Suspicious colonies were picked and dealt with as before.

Method 3

This followed method 2, except that plating after enrichment in M.S.B. was done on B.S.A. as well as S.S.S.A., increasing the total plates to be examined to 14. To conserve media and time, suspicious colonies were streaked on MacConkey agar only. Slide-agglutination tests with appropriate antisera were carried out and only on positive colonies were confirmatory biochemical tests done.

Method 4

This followed method 3 but two parallel series of M.S.B. in double-strength final concentration (Harvey & Price, 1964) were used. The additional set of broths contained sufficient streptomycin sulphate to yield a final concentration equivalent to 8 μg./ml. of streptomycin base.

Since there was only one swab, duplication here was impossible and therefore the swab in its remaining free fluid was cultured in double-strength M.S.B. without streptomycin. These modifications increased the number of plates to be examined from 14 to 22 per swab. The addition of streptomycin to double-strength M.S.B. to

a concentration of 5 μg./ml. was recommended by B. Moore (personal communication) to enhance the selectivity of that medium for the Aberdeen strain of *S. typhi*. Further investigation in this laboratory showed that the concentration of streptomycin could be increased to 8 μg./ml. without detriment to the isolation of the Aberdeen strain of *S. typhi*.

Minor modifications of technique were introduced from time to time during all methods. Thus, the plate spreading described gave way in the last months of method 1 to spreading for separate colonies with a bacteriological loop. This in itself improved the isolation rate and was therefore used throughout the remainder of the programme.

RESULTS

Method 1 techniques were used to examine 1063 swabs over a period of about one year. Swabs were brought to the laboratory at a rate which finally reached approximately 30 per week. At the end of this period *S. typhi* had been isolated from 60 of the 1063 swabs. Table 1 gives the over-all comparative success in swab positivity of each procedure, direct and indirect, incorporated in method 1.

Table 1. *Analysis of* S. typhi *isolations using method* 1

Total swabs examined	1063
Total swabs positive	60
(*a*) Swabs positive if each technique had been used alone:	
Direct culture on S.S.S.A. of swab fluid and dilutions	20
Direct culture on B.S.A. of swab fluid and dilutions	15
Direct culture on S.S.S.A. of centrifuged swab fluid deposit	17
Direct culture on B.S.A. of centrifuged swab fluid deposit	15
After enrichment of swab fluid and dilutions with recovery on S.S.S.A.	50
After enrichment of swab fluid and dilutions with recovery on B.S.A.	11
(*b*) Swabs positive by one or more techniques:	
Swabs positive by direct culture only	8
Swabs positive after enrichment only	21
Swabs positive by both direct culture and after enrichment	31
Direct cultures positive on B.S.A. only	3
Direct cultures positive on S.S.S.A. only	4
Direct cultures positive on both media	1
Cultures after M.S.B. enrichment positive on	
B.S.A. only	1
S.S.S.A. only	19
Both media	1

In assessing the results, it was apparent that success, in the main, had been obtained in the enrichment cultures with S.S.S.A. as recovery medium. Of the 10 positive swabs in the first 450, eight were positive on S.S.S.A. after enrichment only, one was positive after enrichment on both S.S.S.A. and B.S.A. and one by direct plating on S.S.S.A. as well as on both S.S.S.A. and B.S.A. after enrichment. No swab was positive by direct plating on B.S.A.

Spreading for single colonies was now introduced. Of the next 525 swabs in method 1, 30 were positive. Although direct plating on S.S.S.A. and enrichment followed by S.S.S.A. were still more productive, positive results with B.S.A. for

direct plating now began to appear but, of the eight instances of positivity with B.S.A., only in one swab was this the only method positive.

The disappointing results with B.S.A. led to closer attention being paid to its preparation. It was found that, for best results, the medium had to be made in volumes no greater than 500 ml., required constant swirling during preparation to give uniform dispersal of the flocculent precipitate, required cooling to 50° C. with continued swirling even during pouring into plates and was best used with a dry surface and with minimal delay.

The remaining 88 swabs of the 1063 examined by method 1 yielded 20 which were positive. A more even distribution of positivity was now apparent, 14 swabs were positive using both S.S.S.A. and B.S.A. media. In a further three the credit for recovery goes to B.S.A., two of these were positive by direct culture and the third on B.S.A. after enrichment in M.S.B.

Table 2. *Analysis of* S. typhi *isolations using method* 2

Total swabs examined	45
Total swabs positive	15
(*a*) Swabs positive if each technique had been used alone:	
Direct culture on B.S.A. of swab fluid and dilutions	14
After enrichment of swab fluid and dilutions with recovery on S.S.S.A.	3
(*b*) Swabs positive by one or more techniques:	
Swabs positive by direct culture on B.S.A. only	12
Swabs positive after enrichment on S.S.S.A. only	1
Swabs positive by both direct culture and after enrichment	2

In examining B.S.A. plates difficulty still arose from the generalized blackening of the medium due to vigorous production of H_2S by sewage organisms. It was suggested by B. Moore (personal communication) that a snugly fitting filter-paper disk, moistened with a weak solution (1–2 %) of lead acetate, should be inserted into the lids of B.S.A. plates. This proved very helpful by removing excess H_2S without any adverse effect on production of black halos by colonies of *S. typhi* or *S. paratyphi B.* This was introduced and used throughout methods 2–4.

In an effort to reduce the amount of work entailed for each swab using method 1, the procedures were cut and only the two which showed most promise were carried over as method 2. By this curtailed method, 45 swabs were examined and 15 were positive for *S. typhi.* In this series, B.S.A. certainly outscored M.S.B. enrichment followed by S.S.S.A. Only one positive would have been missed had the latter been omitted (Table 2).

Method 3 closely followed method 2 but plating after enrichment on both B.S.A. and S.S.S.A. was done for comparison of efficacy. A total of 72 swabs was covered by method 3 and 32 were positive. If B.S.A. had been the only solid medium used, 28 swabs would have been positive leaving only four which were positive only on S.S.S.A. after enrichment. Again, of the 28, four were positive on the B.S.A. only after M.S.B. enrichment (Table 3).

In the gross, however, direct cultures on B.S.A. were positive in 24 swabs, enrichment followed by B.S.A. in 14 and enrichment followed by S.S.S.A. in 15.

These results indicated a necessity to retain the use of S.S.S.A. as a recovery medium after enrichment, even although B.S.A. was also to be used, so that the isolation rate might be as high as possible.

Table 3. *Analysis of* S. typhi *isolations using method* 3

Total swabs examined	72
Total swabs positive	32
(*a*) Swabs positive if each technique had been used alone:	
Direct culture on B.S.A. of swab fluid and dilutions	24
After enrichment of swab fluid and recovery on B.S.A.	14
After enrichment of swab fluid and recovery on S.S.S.A.	15
(*b*) Swabs positive by one or more techniques:	
Swabs positive by direct culture on B.S.A.	11
Swabs positive after enrichment only	8
Swabs positive by both direct culture and after enrichment	13
Cultures after M.S.B. enrichment positive on	
B.S.A. only	4
S.S.S.A. only	4
Both media	0

Table 4. *Analysis of* S. typhi *isolations using method* 4

Total swabs examined	110
Total swabs positive	67
(*a*) Swabs positive if each technique had been used alone:	
Direct culture of swab fluid and dilutions on B.S.A.	33
After enrichment of swab fluid in double-strength M.S.B. with:	
Recovery on B.S.A.	28
Recovery on S.S.S.A.	25
After enrichment of swab fluid in streptomycin double-strength M.S.B. with:	
Recovery on B.S.A.	40
Recovery on S.S.S.A.	36
(*b*) Swabs positive by one or more techniques:	
Swabs positive by direct culture on B.S.A. only	6
Swabs positive after enrichment only	34
Swabs positive by both direct culture and after enrichment	27
Cultures after double-strength M.S.B. enrichment positive on:	
B.S.A. only	4
S.S.S.A. only	0
Both media	0
Cultures after streptomycin double-strength M.S.B. enrichment positive on:	
B.S.A. only	8
S.S.S.A. only	4
Both media	3
Cultures after both M.S.B. enrichments both positive on:	
B.S.A. only	3
S.S.S.A. only	1
Both media	2
Other combinations of cultures positive after enrichments only	9

Method 4 yielded 67 swabs positive for *S. typhi* out of 110 examined. Use of streptomycin M.S.B. contributed positives which would not otherwise have been obtained but would have missed positives had it been used alone. Again the use

of S.S.S.A. as a recovery medium after enrichment also contributed positive results which would have been missed if it had not been used (Table 4).

S. paratyphi B was isolated from 186 sewer swabs during the entire programme and other salmonella serotypes were isolated from 42 sewer swabs (Table 5). *S. typhi* and *S. paratyphi B* were recovered together from 12 swabs in the programme but multiple isolations involving other salmonella serotypes did not occur.

Table 5. *Other salmonellas isolated from sewer swabs during programme*

Name	Number of swabs positive
S. paratyphi B	186
S. thompson var. *berlin*	28
S. typhimurium	7
S. infantis	2
S. dublin	3
S. give	1
S. tennessee	1
Total	228

Table 6. *Results obtained from sewers related to known excreters 'A' and 'B'*

Sampling sites	Method 1	Method 2	Method 3	Method 4
Domestic sewer A	19/40	4/4	12/12	20/20
Domestic sewer B	11/22	3/4	7/12	15/22
Road A				
15 in. sewer	1/15 (1)	3/5 (0)	4/11 (0)	10/23 (0)
18 in. sewer	0/6 (0)	2/5 (0)	4/11 (0)	8/23 (0)
21 in. sewer	2/41 (1)	1/6 (0)	Not sampled	Not sampled
Road B				
9 in. sewer	6/16 (8)	1/5 (5)	4/13 (10)	5/20 (10)
15 in. sewer	0/15 (3)	1/6 (4)	1/4 (3)	0/3 (3)

N.B. Numerator indicates number positive for *S. typhi* and denominator total number of swabs. Numbers in parentheses indicate number positive for *S. paratyphi B*.

DISCUSSION

Results obtained by the four methods in this programme are not directly comparable with each other because of variation in the sites sampled during each method. In method 1 a high proportion of sewers sampled were of large diameter and each served large areas of the city. These sewers were sampled to allow tracing back from larger to smaller sewers. Certain smaller sewers related to known excreters were included as technique controls in method 1. Consistent isolations of *S. typhi* were not obtained from large sewers, and even in the smallest sewers results were disappointing. Methods 2–4 arose from efforts to improve isolation rates.

In Table 6 comparison is made of results obtained from certain sites related to known excreters which were sampled by all four methods. Improvement in the reliability of methods is seen, especially in the individual domestic sewers. With larger sewers more than half the swabs were always negative.

Isolation of *S. typhi* from sewage had always been recognized to be difficult. Moore's successful tracing of a *S. typhi* carrier (1950) by the sewer swab technique appears to have been due largely to his use of B.S.A., especially in direct techniques. The relative inefficiency of enrichment cultures was noted and studied by Moore (1948, 1950) and also Pilsworth (1960). The latter used a brilliant green selenite broth with added sulphonamide to give some improvement in enrichment procedure. Harvey & Price (1964) found that selenite broth in double concentration enhanced the isolation rate of *S. typhi* from polluted culvert water. They found the method useful in separating *S. paratyphi B* and *S. typhimurium* from *S. typhi*, and applied it successfully in examining sewage effluent and abattoir lairage washings.

Experience in this programme confirmed the value of B.S.A. in direct culture of sewer swab washings, once initial technical difficulties had been overcome. There appeared to be little to choose between B.S.A. and S.S.S.A. as a recovery medium for use with M.S.B., although in the later stages of the programme B.S.A. was preferred by technical staff because of its greater differentiating power. In method 4, enrichment techniques outscored direct B.S.A. cultures, largely through use of streptomycin with double concentration of M.S.B.

In Table 6 the frequency with which *S. paratyphi B* was isolated from samples from 'B's' larger sewers is shown. This strain frequently outgrew *S. typhi* in enrichment cultures, and occurred occasionally when using streptomycin M.S.B. With direct cultures, the similarity of colonial appearances frequently led to all selected colonies being *S. paratyphi B*. It can be concluded that isolation of *S. typhi* is considerably hampered by the presence of *S. paratyphi B* in sewage. Isolation of *S. typhi* and *S. paratyphi B* together on only twelve occasions during the programme adds further support to this conclusion. Difficulty in isolation of *S. typhi* in the presence of *S. paratyphi B* and other salmonella serotypes was also commented on by Harvey & Price (1964). In considering results from 'A's' sewers, low isolation rates in the larger sewers are attributable to the larger population served by these sewers and greater dilution of *S. typhi*.

The two excreters 'A' and 'B' received several courses of antibiotic therapy during method 1. It is reasonable to attribute some of the poorness of results to the antibiotics because the patient's weekly stool cultures were often negative at these times. 'A' and 'B' differed in their attitude to their infections. 'B', said to be a very particular person, used disinfectant liberally after visiting the toilet, during all methods in the programme. 'A' used disinfectant during method 1 but not during methods 2–4. Some of the negative results from 'B's' domestic sewer may have resulted from this use of disinfectant. The success rate with 'B's' sewers during method 4 was also lowered by 'B's' removal to hospital (and death) 5 weeks before the end of method 4. *S. typhi* was recovered from 'B's' domestic sewer on two occasions up to 10 days after his departure but no further isolations were obtained from 'B's' larger sewers.

S. paratyphi B was isolated at many different sites during the programme. Among the 186 sewer swabs positive for this organism a total of 18 different phage types occurred.

Type 2 was isolated from a sewer draining an institution housing a previously known excreter. Available local records gave no indication of possible sources of other strains. Types Beccles and 3*b* were traced to their sources without difficulty. With each the excreter was an elderly female. Type 3*b* var. 6 was isolated on one occasion from a large sewer some weeks after the Blackpool paratyphoid outbreak due to this strain. Approximately 1000 sewer swabs from our total examined were used in attempts to narrow down the origins of some of the *S. paratyphi B* strains.

CONCLUSION

The results suggested that the generalized swabbing programme in Aberdeen offered little prospect of tracing undetected *S. typhi* excreters. The small size of sewer needing to be sampled would have rendered the programme beyond the resources of all concerned. It is likely that the results were depressed partly by antibiotic therapy, use of disinfectants and by the presence of *S. paratyphi B*. In tracing unknown excreters of *S. typhi* it is less likely that antibiotics or disinfectants would be in use and better results might be attainable, but, in communities of any size, *S. paratyphi B* appears likely to impair results.

On balance, the results support the suggestion (Moore *et al.* 1952) that, in sewer swab surveys, sampling should begin at sewers draining 50 to 100 houses to give reasonable chance of success in tracing *S. typhi* carriers.

SUMMARY

Laboratory aspects of a programme of sewer swabbing during and after the Aberdeen typhoid fever outbreak, 1964, have been described. Results were discussed and reasons suggested for the relative insensitivity of methods employed in the isolation of *Salmonella typhi*. It was tentatively concluded that generalized sewer swabbing in a large city was not likely to be very helpful in tracing undetected excreters of *S. typhi* during and immediately after a major typhoid fever outbreak. The cost of the laboratory side of this investigation, for staff salaries, equipment, media, etc., was somewhat over £7000.

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Vaccination with live type 4 adenovirus: evaluation of antibody response and protective efficacy

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The importance of adenovirus as a cause of disease in military recruits has stimulated interest in the development of effective adenovirus vaccines. Several studies conducted between 1956 and 1960 demonstrated that inactivated adenovirus vaccines were highly effective in preventing adenovirus disease in military recruits (Davenport, 1962). However, in later studies, variation in antigenic potency of different lots of vaccine and failure to obtain consistently the desired high protection rates were noted (Meiklejohn, 1963). Moreover, it was found that inactivated vaccines may be contaminated with simian viruses. The problem of vaccine safety was further complicated by the discovery of oncogenicity of certain adenoviruses and of the phenomenon of 'hybridization' of adenoviruses with the oncogenic simian virus SV–40 (cf. review by Hilleman, 1966). Recent data provided suggestive evidence that the tumorigenic potential of adenoviruses, SV–40 and adenovirus –SV–40 'hybrids' is inactivated by formaldehyde used for vaccine production (Truffelli *et al.* 1967). Although the latter findings are encouraging, they offer no answer to the problems of potency and contamination with simian viruses.

Because of the inadequacy of inactivated vaccines, other approaches to adenovirus immunization were explored. Investigators at the National Institutes of Health of the United States developed a technique of producing selective intestinal infection by feeding adenovirus in enteric-coated capsules (Couch *et al.* 1963). The virus was shed from the intestine, but could not be recovered from the throat. In two field trials in which, respectively, 134 and 360 recruits were infected by this method, the enteric adenovirus vaccine was found to be safe, non-communicable and highly effective in preventing adenovirus disease (Edmondson *et al.* 1966; Gutekunst *et al.* 1967). Furthermore, tests in newborn hamsters showed no evidence of oncogenic capacity of the vaccine strain (Chanock *et al.* 1966).

In order to explore further the oral-enteric approach to control of adenovirus disease it seemed of interest to study the effect of enteric adenovirus vaccine in a different ecological setting. For this reason, an investigation was carried out at a training centre for military recruits at Ossendrecht, The Netherlands. Surveillance of respiratory infections at the centre over a 9-year period has shown epidemics of adenovirus infection to recur annually in the winter and spring. Since 1964 only adenovirus type 4 was found in these epidemics. The present paper reports

findings from three controlled field trials conducted in 1966 and 1967 in which 2628 men were given type 4 adenovirus contained in enteric-coated tablets. The trials were primarily designed to test the effectiveness of the vaccine in preventing febrile and afebrile adenovirus disease and subclinical adenovirus infection. In addition, serological studies were done to acquire information on other points of interest. These included: (*a*) spread of virus from vaccinated men to contacts; (*b*) magnitude of the antibody response to vaccine and to naturally acquired adenovirus infection; and (*c*) significance of vaccine-induced and naturally acquired antibody as indicator of immunity.

MATERIALS AND METHODS

Vaccine

Live, lyophilized adenovirus type 4 (strain CL 68578) contained in an enteric-coated tablet was prepared by Wyeth Laboratories under contract to the Vaccine Development Branch, National Institute of Allergy and Infectious Diseases, and supplied through the courtesy of Dr D. I. Mullally. A detailed account of the history of the vaccine strain and the preparation of the vaccine was given previously (Chanock *et al.* 1966). The quantity of virus present in a tablet was $10^{4\cdot7}$ to $10^{4\cdot9}$ 50 % infectious doses for human diploid cell cultures. A titre of $10^{5\cdot5}$ was found at the Wyeth Laboratories, using human embryonic kidney tissue (Dr H. Tint, personal communication).

Study population and plan of study

The study was conducted in a military training centre at Ossendrecht, The Netherlands. At intervals of 2 months, groups of about 2400 to 2600 recruits entered the camp to receive an 8-week course of basic training. The vast majority of these men arrived on post on the same day. A small proportion (less than 3 %) of the men entered 1 week later. During the period of basic training, no other recruits were introduced into the camp. The recruits were formed into companies of slightly more than 200 men, all of which followed a similar training schedule. The companies were trained separately and were housed in separate dormitory areas. Recruits from the same company ate together. Opportunities for contact between men from different companies were limited by these circumstances to occasional association at the common mess hall and to attendance at the theatre during off-duty hours.

Three separate trials were undertaken. In each trial, the plan and purpose of the study were explained to the recruits before the beginning of the vaccination programme and the men were asked whether they agreed to take part in the study. All recruits volunteered to participate.

The first trial was performed in August and September 1966. During the second week of training two groups comprising, respectively, 86 and 85 recruits were selected by a random process from one of the 11 companies formed. Recruits of the first group were fed a tablet containing type 4 adenovirus, those of the second group a dummy tablet containing only diluent fluid. Blood specimens were

obtained from 75 men of the vaccine group and from 72 men of the control group before administration of vaccine or dummy and, again, 3 and 6 weeks later. In addition, paired serum samples were taken from a randomly selected 10 % of the recruits of each of the 10 remaining companies on the first or second day after arrival in the camp and, again, during the eighth week of training.

The second trial was conducted from mid-January to mid-March 1967. The recruit population at the time comprised 12 companies. Vaccine tablets were fed to all men of four companies and to a randomly selected 50 % of the men of four other companies; the men of the remaining four companies did not receive vaccine. In all, 1283 men were vaccinated, whereas 1357 men were not vaccinated. The vaccine was given within 2 days of arrival in the camp. No vaccine was administered to the men who entered at the beginning of the second week of the training period. Hence, the size of the vaccinated group was slightly smaller than that of the control group. All unvaccinated recruits were given tablets containing aspirin, phenacetin and caffeine. The appearance of these tablets was similar to that of the vaccine tablets. None of the recruits was informed whether vaccine or a tablet containing no virus was given to him. Similarly, the clinical observers were unaware of the vaccination status of men reporting at sick call. Blood specimens were taken from a randomly selected 20 % of vaccinated and unvaccinated recruits on the day of vaccination and, again, 3 and 8 weeks later.

The third trial was performed from mid-March to mid-May 1967 and was similar to the second trial in all respects. The number of men who received vaccine was 1259, whereas 1281 men were not vaccinated.

Criteria for evaluation of vaccine

The antibody response and protective capacity were used as criteria for evaluation of the vaccine. Assessment of the protective capacity was made by comparing the incidence of febrile and afebrile respiratory illness due to adenovirus in vaccinated recruits with that in unvaccinated recruits. The study of the protective effect was restricted to the two groups of recruits who entered service in the winter (second and third trials). Incidence rates were calculated for a 7-week period including the second to eighth week of training. Illnesses occurring in the first week of training were excluded to allow time for the vaccine to demonstrate an effect. Patients who reported to sick call with respiratory illness were considered febrile if the rectal temperature was 38·0° C. (100·4° F.) or higher. These men were admitted to the sick quarters. Patients reporting with respiratory illness associated with a temperature of lower than 38·0° C. were considered afebrile and treated as outpatients. Specimens for laboratory examination were obtained on patients with febrile respiratory illness from all 12 companies and on patients with afebrile respiratory illness from only six companies (two 100 %-vaccinated companies, two 50 %-vaccinated companies and two non-vaccinated companies).

Laboratory methods

Specimens

Specimens for laboratory examination consisted of a throat swab and a sample of blood on the day of report to sick call, and a second sample of blood about 14 days later. Sera from blood specimens were frozen and stored at −20° C. until tested. Immediately after collection, throat swabs were placed in tubes containing 3 ml. of GLY medium (0·5 % gelatin, 0·5 % lactalbumin hydrolysate, and 0·1 % yeast extract in Hanks's balanced salt solution) and were kept at 2–4° C. until processed. The specimens were inoculated into cell cultures within 16 hr. Before inoculation, swabs were squeezed against the glass; the fluids from the swabs were used without previous centrifugation.

Isolation of virus

For virus isolation attempts, 0·5 ml. of fluid was inoculated into a tube culture of diploid fibroblast cells (N–3) derived from human embryonic lung. The N–3 cell strain was established in our laboratory. The cells were grown in Eagle's minimum essential medium and 10 % calf serum. For maintenance, calf serum was replaced by 2·5 % chicken serum. After inoculation, cultures were incubated at 35° C. on a drum rotating 12 times an hour. If no definite cytopathic effect occurred, the tubes were subjected to three cycles of freezing and thawing. The harvest was inoculated into fresh tube cultures. The total incubation period was 30 days. Adenovirus strains were typed by neutralization tests with rabbit antisera against adenovirus prototype strains.

Serological tests

Neutralization tests were carried out in HeLa cell cultures using fourfold dilutions of unheated serum and test doses of virus calculated to contain 3–10 TCID50 on the fourth day of incubation. HeLa cell cultures were grown in a medium consisting of 20 % horse serum and 0·5 % lactalbumin hydrolysate in Hanks's balanced salt solution. For maintenance, horse serum was replaced by 3 % rabbit serum. The virus used was the prototype strain of adenovirus type 4. Serum dilution and virus were incubated at 37° C. for 60 min. The mixture was then transferred to duplicate tube cultures. The tests were read on the fourth day of incubation. The titres were based on initial serum dilution before addition of other reagents and were expressed as the reciprocal of the highest serum dilution producing complete inhibition of cytopathic effect in both tubes, the fourfold higher dilution showing cytopathic effect in both tubes. If the higher dilution produced complete inhibition in one of the two tubes, the titre was recorded as being intermediate between the two dilutions.

Complement-fixation (CF) tests were done in microtitre plates according to the technique described by Sever (1962). Antigen consisted of a suspension of the prototype strain of type 4. In all serological tests, sera from a single person were always titrated simultaneously.

RESULTS

Antibody response to the vaccine

Frequency

In each of the three trials, sera from randomly selected vaccinated men and controls were tested for neutralizing and CF antibody. The frequency of presence of neutralizing antibody in the two groups of men was comparable at the start of the study. Initial antibody was found in 29 out of 75 (39 %), 61 of 170 (36 %) and 55 of 167 (33 %) of vaccinated men in the first, second and third trials respectively. The corresponding figures for the controls were 78 of 244 (32 %), 64 of 203 (32 %) and 69 of 208 (33 %).

Table 1. *Neutralizing antibody response in initially sero-negative recruits* 3 *and* 8 *weeks after feeding of type* 4 *adenovirus*

Trial	Group	No. of men studied	Percentage sero-positive	
			3 weeks	8 weeks*
1	Vaccine	46	85	85
	Control (50 %-vaccinated company)	49	8	8
	Control (non-vaccinated companies)	117	—†	4
2	Vaccine	109	77	64
	Control (50 %-vaccinated companies)	43	16	19
	Control (non-vaccinated companies)	96	9	14
3	Vaccine	112	87	78
	Control (50 %-vaccinated companies)	45	11	62
	Control (non-vaccinated companies)	94	9	70

* In the first trial sera were collected at 6 weeks after vaccination.
† No sera collected.

Table 1 summarizes the results of neutralization tests in recruits without pre-existing neutralizing antibody. It is seen that 77–87 % of the recruits showed neutralizing antibody at 3 weeks after vaccination. During the following 5 weeks the percentages of sero-positive men declined slightly. The mean antibody titres, not shown in the table, decreased also slightly during this period. The CF test appeared to be much less effective in detecting an antibody response to vaccine than the neutralization test. The percentages of recruits who showed a significant (fourfold or greater) rise in CF antibody at 3 weeks following vaccination were 24 in the first trial, 36 in the second trial and 43 in the third trial.

Eight to 16 % of the controls developed neutralizing antibody during the first 3 weeks of the training period. Development of antibody was detected slightly more frequently in controls of 50 %-vaccinated companies than in those of entirely untreated companies. The differences were not statistically significant ($P > 0{\cdot}25$, χ^2 test).

Data on the antibody response to vaccine in recruits with pre-existing neutralizing antibody are presented in Table 2. The response appeared to be related to the level of pre-existing antibody. An antibody rise was observed most often in recruits with a low initial antibody titre and least often in men with a high titre.

Magnitude

It seemed of interest to compare the magnitude of the antibody response to the vaccine with that to naturally acquired adenovirus infection. Convalescent sera from initially sero-negative recruits with febrile and afebrile respiratory illness from whom adenovirus type 4 was recovered were tested for content of neutralizing antibody. In addition, postinfection sera from controls who showed

Table 2. *Influence of pre-existing neutralizing antibody upon antibody response to feeding of type 4 adenovirus*

Antibody titre before vaccination	No. of recruits studied	Neutralizing antibody rise (fourfold or greater) after vaccination	
		No.	%
4–8	33	26*	79
16–32	61	32	52
64–128	48	11	23
≥256	4	0	0

* $P < 0{\cdot}001$ (χ^2 test).

Table 3. *Magnitude of the neutralizing antibody response in initially sero-negative recruits to feeding of type 4 adenovirus and to naturally acquired adenovirus infection*

Group	No. of men studied	No. of men with indicated antibody titre						Geometric mean antibody titre
		4–8	16–32	64–128	256–512	1024–2048	4096	
Vaccine	221	95	79	36	11	–	–	11
Subclinical adenovirus infection	81	12	23	26	20	–	–	49
Afebrile adenovirus illness	31	1	5	11	8	6	–	137
Febrile adenovirus illness	20	–	1	5	6	6	2	446

serological but no clinical evidence of infection were tested; the sera were collected at the end of the 8-week course of training. To allow comparison of the antibody response to subclinical infection and that to illness, only sera from recruits from one trial, in this case the third trial, were selected. The distribution of antibody titres of these sera and of sera collected from initially sero-negative recruits at 3 weeks after vaccination is shown in Table 3. The postvaccination titres were significantly lower than the titres elicited by naturally acquired subclinical infection ($P < 0{\cdot}001$, two-sided Wilcoxon's two-sample test). Furthermore, it appeared that the magnitude of the antibody response to infection was related to the quality of the clinical response ($P < 0{\cdot}001$, k sample trend test, van Eeden & Rümke, 1961). Antibody titres attained in febrile illness were higher than those

found in afebrile illness. The antibody response to afebrile illness in turn was greater than that to subclinical infection.

Protective effect of the vaccine

Total respiratory illness

Tables 4 and 5 present a summary of the evaluation of the vaccine in the second and third trials conducted in the winter of 1967. Adenovirus was responsible for less than one-fifth of the cases of febrile and afebrile respiratory illness in

Table 4. *Incidence of febrile and afebrile respiratory illness caused by adenovirus in vaccinated and unvaccinated recruits in second trial*

	Vaccination state of companies					
	100%	Nil	50%		Totals	
	Vaccine	Control	Vaccine	Control	Vaccine	Control
	Febrile respiratory illness					
No. in group	878	889	405	468	1283	1357
All illnesses:						
Rate/1000	59	35	22	43	48	38
Adenovirus illness:						
No. tested for virus	40	26	6	17	46	43
No. positive	0	3	0	5	0*	8*
Est. rate/1000†	0	4	0	13	0	7
	Afebrile respiratory illness					
No. in group	455	400	211	226	666	626
All illnesses:						
Rate/1000	336	368	313	460	329	401
Adenovirus illness:						
No. tested for virus	110	76	35	45	145	121
No. positive	2	9	0	6	2‡	15‡
Est. rate/1000	6	44	0	61	5	50

* $P < 0{\cdot}01$ (χ^2 test).

† Estimated rate calculated by applying the percentage of virus positive cases to the rate of all illnesses.

‡ $P = 0{\cdot}01$ (χ^2 test).

controls from the second trial, and for about one-third of the cases of afebrile respiratory illness in controls from the third trial. It is obvious, with the inclusion of many cases of respiratory disease of other etiology, that a comparison of all cases of illness in vaccinated men and controls affords a rather insensitive index of the protective effect of the vaccine. Thus, it was not surprising to find that total afebrile respiratory illness was only slightly reduced in the vaccinated groups.

The incidence of febrile respiratory illness in vaccinated recruits from the second trial appeared to be higher than that in unvaccinated men. This was attributable to a marked difference in incidence between recruits of totally vaccinated and unvaccinated companies. It seems unlikely that vaccination was

responsible for this effect since most patients with febrile respiratory illness in the vaccinated group were admitted during the last 4 weeks of the training period and no adenovirus was recovered from the oropharynx of the patients at the time of illness. Possibly, the outcome was affected by variation in activity of respiratory agents and in reporting to sick call between different companies.

Table 5. *Incidence of febrile and afebrile respiratory illness caused by adenovirus in vaccinated and unvaccinated recruits in third trial*

	Vaccination state of companies					
	100%	Nil	50%		Totals	
	Vaccine	Control	Vaccine	Control	Vaccine	Control
	Febrile respiratory illness					
No. in group	847	820	412	461	1259	1281
All illnesses:						
Rate/1000	33	63	29	33	32	52
Adenovirus illness:						
No. tested for virus	17	41	9	8	26	49
No. positive	0	23	1	7	1*	30*
Est. rate/1000†	0	35	3·2	29	1·2	32
	Afebrile respiratory illness					
No. in group	431	470	210	242	641	712
All illnesses:						
Rate/1000	179	294	248	186	201	257
Adenovirus illness:						
No. tested for virus	48	90	39	32	87	122
No. positive	1	35	3	10	4‡	45‡
Est. rate/1000	3·7	114	19	58	9	95

* $P < 0{\cdot}001$ (χ^2 test).

† Estimated rate calculated by applying the percentage of virus positive cases to the rate of all illnesses.

‡ $P < 0{\cdot}001$ (χ^2 test).

Total febrile respiratory illness was markedly (about 40 %) reduced in vaccinated men from the third trial who underwent training between mid-March and mid-May. During this period about 60 % of febrile respiratory illnesses were due to adenovirus.

Adenovirus illness

The protective effect of the vaccine is clearly shown when the incidence of adenovirus illness in the vaccinated and unvaccinated recruits is compared (Tables 4 and 5). Diagnosis of adenovirus illness was based on recovery of virus from the throat. All adenovirus strains isolated were found to be of type 4. In each of the two trials conducted in the winter of 1967 vaccination effected a significant reduction in the number of patients with febrile and afebrile adenovirus illness.

The incidence of adenovirus illness was estimated by applying the rate of virus-

positive cases to the total illness rate. In the second trial the estimated rates for febrile adenovirus illness were 0 per 1000 in the vaccinated recruits and 7 per 1000 in the unvaccinated; the corresponding rates for afebrile adenovirus illness were 5 and 50 per 1000. This represented a complete reduction in incidence of febrile adenovirus illness and about 90 % reduction in incidence of afebrile adenovirus illness. The estimated reductions in incidence of adenovirus illness in the third trial were about 96 % for febrile illness and about 91 % for afebrile illness.

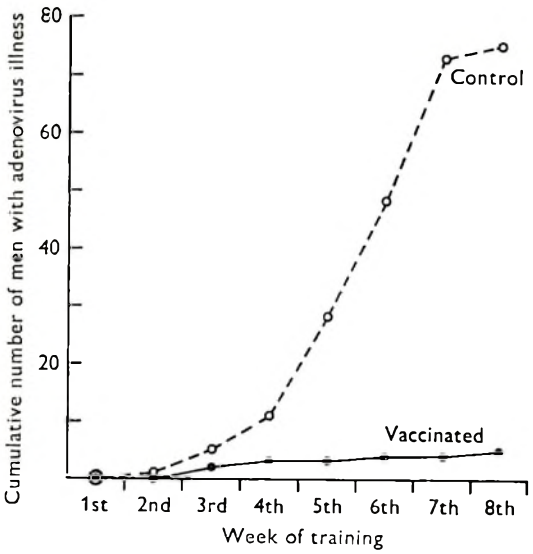

Fig. 1. Cumulative number of recruits with febrile and afebrile adenovirus illness in third trial.

The distribution of adenovirus illness in the vaccinated and unvaccinated groups from the third trial by week of training is shown in Fig. 1. The greatest number of illnesses occurred in the fifth, sixth and seventh weeks of training.

Antibody status and subsequent infection

To obtain information on the relation between antibody status and susceptibility to subsequent adenovirus infection, sera taken from vaccinated and unvaccinated recruits from the third trial at 3 weeks after their entry into the training centre were tested for presence and level of neutralizing antibody. The serological data were then correlated with the infection patterns of the recruits. Clinical as well as subclinical infections were included. Diagnosis of infection was based on significant rises in antibody titre occurring between the third and eighth week of training. The 5-week interval corresponded to the period in which the majority (over 90 %) of adenovirus illness occurred.

As seen in Table 6, there was in both groups an inverse relationship between the titre of pre-existing neutralizing antibody and the frequency of serologically detected adenovirus infection ($P = 0{\cdot}01$, k sample trend test; van Eeden & Rümke, 1961). Infection was observed most often in recruits who were devoid of

antibody and least often in individuals with high levels of pre-existing antibody. Antibody stimulated by vaccination appeared to be indicative of a higher degree of protection than that produced by naturally occurring infection. Vaccinated recruits with antibody titres of 4 to 32 were infected significantly less often than were controls with the same antibody titres. A similar difference was found when vaccinated and unvaccinated recruits without pre-existing antibody were compared. The latter finding suggests that enteric infection with adenovirus type 4 is capable of providing protection without concomitant production of detectable antibody titres.

Table 6. *Influence of vaccine-induced and naturally acquired neutralizing antibody upon susceptibility to clinical and subclinical adenovirus infection*

Group	Neutralizing antibody titre at 3 weeks after onset of training	No. of recruits studied	No. showing antibody rise between 3rd and 8th week of training	
			Neutralization	CF
Vaccine*	<4	13	3†	2‡
	4–8	39	6	1
	16–32	34	2 §| 0	
	⩾64	26	0	0
	Totals	112	11	3
Control	<4	129	79†	71‡
	4–8	21	8	4
	16–32	37	10 §| 4	
	⩾64	22	1	1
	Totals	209	98	80

* Sero-negative. † $P = 0{\cdot}02$ (χ^2 test). ‡ $P = 0{\cdot}01$ (χ^2 test).
§ $P = 0{\cdot}01$ (χ^2 test). || $P = 0{\cdot}01$ (χ^2 test).

Subclinical adenovirus infection

From the data obtained in the third trial, it was possible to study the effectiveness of the vaccine in preventing subclinical adenovirus infection. The study was confined to the six companies surveyed for febrile and afebrile adenovirus illness. The purpose of the investigation being to measure the protective effect of the vaccine against subclinical adenovirus infection alone, recruits with febrile and afebrile respiratory illness from whom adenovirus was isolated were set aside. A comparison was made of the frequency of serologically detected adenovirus infections occurring in vaccinated and unvaccinated recruits of the remaining population during the last 5 weeks of training.

The neutralization test showed increases in titre in 8 % of 134 vaccinated recruits and in 40 % of 110 controls. The corresponding percentages for the CF test were 2 and 33. Since the presence of pre-existing antibody might prevent serological detection of subsequent adenovirus infection, the comparison of the frequency of serologically detected infections between vaccinated and unvaccinated recruits was restricted to those without pre-existing neutralizing or CF antibody. Neutralizing antibody rises were then found in 1 of 10 vaccinated men and in 34 of

61 controls. The difference was significant ($P = 0{\cdot}02$, χ^2 test) and represented about 82 % reduction. CF antibody rises were detected in three of 22 vaccinated men and in 26 of 56 controls ($P = 0{\cdot}01$, χ^2 test), giving about 71 % reduction.

DISCUSSION

Outbreaks of adenovirus infection among recruits at Ossendrecht usually develop in the second month of training, whereas in most military training centres in the United States adenovirus infection commonly occurs during the first weeks of training (Hilleman, 1957; Miller *et al.* 1965). It seems likely that the pattern of infection is related to the method of recruitment. Dutch recruits are admitted non-continuously at intervals of 2 months, whereas there is a continuous influx of fresh recruits in training centres in the United States. The late occurrence of adenovirus illness in recruits at Ossendrecht affords a special benefit in vaccination studies in that the protective effect of vaccine may be obtained before the beginning of an outbreak. Enteric type 4 adenovirus vaccine appears to be highly effective in the ecological setting of the present study. The vaccine provided almost complete protection against febrile adenovirus illness and slightly less protection against afebrile adenovirus illness. In addition, about 70 % reduction in subclinical adenovirus infection was found as a result of vaccination. No adverse reaction to the vaccine came to our attention. The prophylactic effect of the vaccine on total respiratory illness was less spectacular. This is explained by the fact that a large proportion of respiratory illnesses were not caused by adenovirus.

In contrast to the excellent protective capacity, the ability of the vaccine to produce antibody was poor. The vaccine was capable of providing protection even without concomitant production of detectable antibody. These findings point to the limited significance of the presence of antibody and the height of antibody titres as measures of the existence and degree of immunity. The demonstration that low antibody titres stimulated by vaccination were associated with a higher degree of protection than were the same titres of antibody produced by naturally acquired infection also points that way. Apparently, the antibody-stimulating effect of enteric infection with adenovirus type 4 was only remotely related to the protective capacity of such infection. It seems unlikely that the poor antibody response to vaccination was due to lack of sensitivity of the neutralization test employed. In comparative tests with sera from recruits with naturally acquired adenovirus type 4 infection high antibody titres were found. Similarly, in a previous study of adenovirus immunization in infants we observed high antibody titres after administration of two or three doses of inactivated vaccine, using essentially the same neutralization technique (van der Veen *et al.* 1967).

In previous studies enterically administered type 4 adenovirus did not spread to susceptible contacts (Chanock *et al.* 1966; Edmondson *et al.* 1966; Gutekunst *et al.* 1967). We found that a small proportion of unvaccinated controls without pre-existing neutralizing antibody developed antibody during the first 3 weeks of the training period. Antibody rises were detected slightly more frequently in controls of 50%-vaccinated companies who were in close personal contact with

vaccinated recruits than in men of entirely untreated companies who had less or no contact with vaccinated men. The differences were not statistically significant. Although these findings do not afford unequivocal evidence of lack of communicability of enteric type 4 infection, they nevertheless suggest that, if spread of virus occurs at all, virus is transmitted only with difficulty.

SUMMARY

Live type 4 adenovirus contained in enteric-coated tablets was given to 2628 military recruits. In two trials, the enteric adenovirus vaccine reduced febrile and afebrile adenovirus illness by about 90% or more. Moreover, a decided protective effect against subclinical adenovirus infection as evidenced by seroconversion was found.

The antibody-stimulating effect of enteric adenovirus vaccine was not an adequate measure of its protective capacity. The neutralizing antibody response of recruits given vaccine was poor in comparison with the antibody response to naturally acquired infection with type 4 adenovirus. The latter response was related to the severity of infection.

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Genetic marker studies on poliovirus type 1 strains from the Blackburn poliomyelitis outbreak in 1965

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In July 1965 an outbreak of poliomyelitis developed in Blackburn, a town in north-west England with a population of about 100,000. At the time some 52% of the inhabitants aged less than 40 years had received three or more inoculations of inactivated poliomyelitis vaccine (Salk). No live attenuated vaccine had been used (Ardley, 1966).

The first patient was a girl of 18 who died of encephalitis on 8 July after an illness lasting 12 days. Poliovirus type 1 was isolated from the brain and faecal material collected at autopsy. Ten days after the death of the first patient a 4-year-old child became ill and in the next 2 weeks five more paralytic cases developed. On 3 August vaccination of the entire community with trivalent oral vaccine was begun.

The epidemiological picture then became less clear. Notification of cases continued but these were largely non-paralytic. On clinical grounds 53 patients were considered to have had poliomyelitis.

Specimens of faeces for virus investigation were collected from many of the cases, suspect cases and their contacts. In the first instance they were examined in the Preston Public Health Laboratory (Robertson *et al.* 1966). Poliovirus type 1 alone was isolated from six of the 53 patients, Coxsackie B 5 virus alone in two and poliovirus other than type 1 alone from 12, the remaining 33 being negative. In addition, many faecal specimens from other suspect cases or contacts of cases were examined, and 160 of these contained poliovirus type 1 alone or in combination with other viruses. Two specimens contained Coxsackie B 5 virus, two others Coxsackie B 3 virus and one echovirus type 8. All of these specimens were collected after the beginning of the mass vaccination campaign.

The faecal extracts from which poliovirus type 1 had been isolated were sent from the Preston Public Health Laboratory to the Virus Reference Laboratory for fuller examination with a view to differentiation into naturally occurring and vaccine strains by marker tests.

Marker tests. Three genetic marker tests were used:

(*a*) The reproductive capacity temperature marker (R.C.T.) which compares the growth of a strain at 39·8° C. with that at 36° C.

(*b*) The dextran inhibition test which measures the degree of inhibition of the strain by 0·05% dextran sulphate.

(*c*) The modified Wecker test which compares the degree of neutralization of

the strain by 'specific' anti-Sabin I serum with that by anti-Mahoney serum in a plaque reduction assay.

The values expected for naturally occurring and vaccine prototype strains are given in Table 1.

Table 1. *Results of marker tests on prototype polio* 1 *strains*

(Average of 5 determinations; range given in parentheses.)

Strain	R.C.T. 39·8° C. $\log_{10}$ reduction	Dextran inhibition $\log_{10}$ reduction	Serological index
Sabin 1	6·0 (6·5–5·5)	2·5 (1·5–3·0)	1·9 (1·0–∞)
Mahoney	0·5 (1·0–+0·5)	0·3 (+0·5–−0·5)	0·1 (0–0·7)

$$\text{Serological index} = \frac{\%\text{ plaque reduction under anti-Sabin 1 serum}}{\%\text{ plaque reduction under anti-Mahoney serum}}.$$

Table 2. *Results of marker tests on cases from which polio* 1 *was isolated*

Case no.	Strain no.	Titre 36° C. $\log_{10}$ TCD 50/0·1 ml.	R.C.T. 39·8° C. $\log_{10}$ reduction	Dextran inhibition $\log_{10}$ reduction	Serological index
1	944 (ex brain)	5·5	5·0	0·5	0·8
	967 (ex faeces)	5·5	5·0	0·5	0·5
2	983	6·0	6·0	0	0·5
3	966	6·0	6·0	0	0·5
4	982	7·0	5·5	0	0·7
5	Failed re-isolation	—	—	—	—
6	1848	6·0	5·0	+0·5	0·6

Table 3. *Results of marker tests where polio type* 1 *alone was isolated from faecal extract*

Strain no.	Titre 36° C. $\log_{10}$ TCD 50/0·1 ml.	R.C.T. 39·8° C. $\log_{10}$ reduction	Dextran inhibition $\log_{10}$ reduction	Serological index
1833	6·5	6·5	−0·5	1·0
1842 C	5·0	5·0	−1·0	n.d.
1846 A*	6·0	5·5	+1·0	0·1
1847 A	7·0	7·0	−0·5	1·4
B	4·5	4·5	−0·5	1·3
C	5·5	5·5	−1·0	1·2
1848†	5·0	5·0	+0·5	0·6
1852	4·0	4·0	0	1·1
1874	6·0	6·0	−0·5	1·1

* Contact of case no. 7.
† Additional strain from case no. 7.
n.d. = not done.

Table 4. *Marker tests on strains isolated from family contacts of the index cases*

Case no.	Contact strain no.	Type	Titre 36° C. $\log_{10}$ TCD 50/0·1 ml.	R.C.T. 39·8° C. $\log_{10}$ reduction	R.C.T. 39·2° C. $\log_{10}$ reduction	Dextran inhibition $\log_{10}$ reduction	Serological index
1	None traced	—	—	—	—	—	—
2	(*a*) 1877 A	Mixture	4·0	n.d.	4·0	—	—
	1877 B	Mixture	4·0	n.d.	4·0	—	—
	1877 C	Mixture	4·0	n.d.	4·0	—	—
	963	Polio 1	6·0	5·0	n.d.	−0·5	0·8
	(*b*) 1878	No growth	—	—	—	—	—
	964	Polio 1	6·0	5·0	n.d.	0	0·8
	222	Mixture	5·0	n.d.	5·0	—	—
3	(*a*) 1863	Mixture	6·0	n.d.	5·0	—	—
	(*b*) 1965	Polio 1	6·0	5·0	n.d.	0	0·6
4	(*a*) 1860	Mixture	5·0	n.d.	5·0	—	—
	(*b*) 1867	Polio 1	6·0	6·0	n.d.	+0·5	n.d.
	(*c*) 1884	Mixture	5·0	n.d.	4·0	—	—
	223	Mixture	5·0	n.d.	5·0	—	—
	(*d*) 205	Mixture	5·5	n.d.	5·0	—	—
	(*e*) 1875	Mixture	5·0	n.d.	5·0	—	—
5	(*a*) 1883	Mixture	5·0	n.d.	4·0	—	—
	(*b*) 1885	Mixture	4·0	n.d.	4·0	—	—
6	225	Mixture	5·0	n.d.	5·0	—	—
7	(*a*) 1866	Mixture	5·0	n.d.	5·0	—	—
	(*b*) 1846	Polio 1	6·0	6·0	n.d.	+1·0	0·1
	(*c*) 1869	No growth	—	—	—	—	—
	(*d*) 228	Polio 1	6·0	6·0	n.d.	0·7	0·7

n.d. = not done; — = not applicable.

RESULTS

The six strains from paralytic cases were all similar and of naturally occurring type (Table 2). They share the peculiarity of being severely inhibited at 39·8° C., a temperature at which most naturally occurring strains grow readily. At 39·2° C., however, the Blackburn strains grow to within 10^{-1} TCD50 of their titre at 36° C. This feature has also been observed in a proportion of type 1 strains isolated from paralytic cases before the introduction of vaccination against poliomyelitis (Cossart, 1967*a*).

From six further cases mixtures of polioviruses, including type 1, were isolated. Since the titres of all these mixtures were reduced by 10^4 TCD 50/0·1 ml. or more at 39·2° C. they are considered to be of vaccine origin.

Table 5. *Comparison of strains from Blackburn with those from Hyde*

Strain no.	Diagnosis	Titre 36° C. $\log_{10}$ TCD50/0·1 ml.	R.C.T. 39·8° C. $\log_{10}$ reduction	Dextran inhibition $\log_{10}$ reduction	Serological index
		Blackburn			
944, 967	Fatal bulbar palsy	5·5	5·0	−0·5	0·8
		5·5	5·0	−0·5	0·5
963	Contact	6·0	5·0	−0·5	0·8
964	Contact	6·0	5·0	0	0·8
965	Contact	6·5	5·0	0	0·6
966	Paralytic	6·0	6·0	0	0·5
981	Non-paralytic	6·5	4·0	−0·5	0·5
982	Non-paralytic	7·0	5·5	0	0·7
983	Non-paralytic	6·0	6·0	−0·5	0·8
		Average 5·0		−0·3	0·7
		Hyde			
1226	Contact	6·0	5·5	−1·0	0·3
1227	Contact	6·5	5·5	−0·5	0·3
1228	Contact	6·5	2·0	−0·5	0·4
1229	Contact	6·5	3·0	−0·5	0·6
1230	Fatal case	6·0	0·5	−0·5	0·4
1231	Contact	6·5	2·0	−1·0	0·7
1232	Non-paralytic	6·5	0	0	0·6
1233	Paralytic	5·5	0·5	0	0·1
1234	Contact	7·0	0·5	0	0·7
1235	Contact	7·0	0·5	0	0·6
1236	Contact	6·5	2·5	−0·5	0·8
1237	Fatal case	6·5	2·0	−0·5	0·8
		Average 2·0		−0·3	0·5

The 160 other faecal samples which had been found to contain poliovirus type 1 were examined. Of these seven were finally found to contain only type 1 virus, and marker tests performed on them showed five to be of vaccine type and two of the same naturally occurring type as was isolated from the index cases (Table 3). On consulting the key these were found to be an additional strain from one of the index cases and a strain isolated from one of his family contacts.

Mixtures of strains from 42 of the 153 remaining specimens were tested for their capacity to grow at 39·2° C. Of these 29 failed to grow at all, eight reached titres of between 10^2 and 10^4 TCD50/0·1 ml., while the growth of the other four mixtures was unaffected. These four contained type 3 poliovirus. Since Sabin 3 vaccine strain multiplies readily at 39·2° C. it is probable that all these strains are of vaccine origin.

All strains from the family contacts of the index cases were examined (Table 4). It seems likely that most of these specimens were collected after the administration of vaccine as the proportion of wild strains is low. As was also observed in the 1962 outbreak in Cardiff (Cossart, 1967*b*) attenuated vaccine does not appear to displace an established wild strain even in an asymptomatic subject.

A point of some interest is that a small outbreak of poliomyelitis due to type 1 virus occurred in the nearby town of Hyde almost simultaneously. The strains from this outbreak could be differentiated from the Blackburn strains by their ability to grow at 39·8° C. (Table 5).

DISCUSSION

The most striking feature of this study is that strains of naturally occurring type were found only in the index cases and their immediate contacts. This is probably because few samples could be collected before the area was saturated with attenuated vaccine, and it emphasizes the effectiveness of this blanketing technique in limiting the spread of wild virus. However, the emergence of other enteroviruses in these circumstances is unexpected and suggests that the production of high titres of homotypic antibody in the community is a component in the mode of action of the vaccine blanket, which may be as significant as its competitive effect.

The R.C.T. test with the upper temperature adjusted to the highest level at which growth of the prototype strain for the outbreak is unaffected seems a useful approach to the problem of mixtures, but some growth of type 3 vaccine strains must be expected up to about 40° C.

These findings illustrate the usefulness of marker tests, not only in differentiating naturally occurring and vaccine strains, but also in characterizing strains from different outbreaks.

SUMMARY

Genetic marker tests were performed on 61 strains of poliovirus isolated during the Blackburn outbreak from cases or suspected cases and their contacts. The results were correlated with the epidemiological data and good agreement was found for the serological and dextran inhibition tests. The reproductive capacity temperature marker, however, showed inhibition at 39·8° C. of strains otherwise judged to be of naturally occurring type. These strains could be differentiated from those of vaccine origin because they grew readily at 39·2° C. This feature also distinguished the strains isolated in Blackburn from those obtained in the nearby town of Hyde in a simultaneous outbreak.

Dr J. Ardley, Medical Officer of Health, Blackburn, kindly provided a key which enabled the contacts of cases to be traced; and Dr L. Robertson, Public Health Laboratory, Preston, made available his extensive records as well as the many strains isolated in his laboratory.

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Sero-epidemiological study of reovirus infection amongst the normal population of the Chandigarh area—northern India

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Reoviruses are widely distributed throughout the world, in man and a large number of animal species. Serological evidence of reovirus infection has been reported in non-isolated human communities of many different countries of the world (Taylor-Robinson, 1965) and it has recently been reported even in remote isolated communities such as Hottentots, Eskimos and Micronesians (Brown & Taylor-Robinson, 1966).

Not much is known about the epidemiology of reovirus infection in India. Small numbers of sera were tested by Ramos-Alvarez & Sabin (1956) and Taylor-Robinson (1965), but no definite sero-epidemiological data in different age groups are available to determine its prevalence. The present investigation was undertaken to examine normal persons belonging to different age groups for presence of haemagglutination-inhibiting (HAI) antibodies in their sera against three types of reoviruses with the idea: (i) of studying the incidence of latent or inapparent infection, (ii) of assessing the rate of infection in different age groups, and (iii) of establishing the normal basal titre of HAI antibodies to reoviruses in different age groups.

MATERIALS AND METHODS

Sera

A total of 264 serum samples was collected from apparently normal persons of different age groups.

One hundred and three sera (from the age group 6 months–5 years) and 34 sera (from the age group, > 5–10 years) were collected mainly from healthy children attending a polio-vaccination clinic for the first time at the Institute of Post-Graduate Medical Education and Research, Chandigarh, and also from healthy children of hospital workers.

Twenty-seven sera were collected from children attending a surgical clinic for operation of congenital abnormalities, and from new trainee technicians of the Institute, and also from household servants and relatives of Chandigarh population. These formed the samples from the age group > 10–20 years.

One hundred serum samples of the age group above 20 years came from doctors, nurses, students, technicians and grade IV employees of the Institute. These sera represented a mixed population of different socio-economic status; some came

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from persons of different states of India, who were residing in Chandigarh temporarily.

All sera were stored at −20° C. if they were not tested immediately.

Virus strains

Three prototype strains of reoviruses, type 1 'Lang' strain, type 2 'D 5 (Jones)' strain and type 3 'Abney' strain were kindly supplied by Dr Leon Rosen (Pacific Research Section, National Institute of Allergy and Infectious Diseases, Honolulu, Hawaii).

Culture system and preparation of haemagglutinating antigen

The primary Rhesus monkey kidney cells were grown in monolayer and were maintained in serum-free Eagle's MEM medium as previously described (Pal, Banerjea & Aikat, 1966). The three prototype strains, after four passages in static MK tissue culture tubes and bottles according to the method of Rosen (1964), were passaged for the fifth time in roller drum culture (12 rev./hr.). After complete degeneration of the cell sheet, tissue culture fluid with degenerated cells served as seed virus and haemagglutinating antigen, and was stored at −20° C.

Haemagglutination-inhibition test

Antigen titrations for the prototype strains were made at each passage level with 0·7% human group O red blood cells according to the method of Rosen (1964). The tissue culture fluids at the fourth and fifth passage level were used as antigens in the HAI test, when the HA titres were 1/128, 1/64, and 1/64 for reoviruses type 1, type 2 and type 3 respectively at the fourth passage level, and 1/256, 1/512, and 1/128 for reoviruses types 1, 2 and 3 respectively at the fifth-passage level. To ensure the amount of antigen used in the HAI test during each run, antigen titrations for the three types of viruses were always made on the same day prior to the HAI test. Haemagglutination-inhibition tests were also performed according to the method of Rosen (1964) with the slight modification of using perspex trays instead of tubes. The sera were inactivated at 56° C. for 30 min., and later adsorbed with 25% acid-washed kaolin. Twofold dilutions of sera in 0·2 ml. volumes starting from 1/10 were tested against 4 units of HA antigen contained in 0·2 ml. using unit volume (0·2 ml.) of 0·7% human group 'O' red blood cells. The dilution of serum showing partial haemagglutination was taken as the end point.

RESULTS

Incidence of reovirus infection

A positive haemagglutination-inhibition at 1/10 or higher was considered as evidence of infection according to the method followed by Rosen (1964). The results of the HAI test in Table 1 showed that infection with reoviruses was not infrequent in India. It was found that antibodies to reoviruses irrespective of their types and multiplicity were present in 191 sera out of 264 sera tested (72%).

Amongst these 19 (7 %) had antibodies against reovirus type 1, 9 (4 %) against type 2, and 56 (21 %) against type 3. Antibodies to any two types and to all three types were also recorded (table 1).

Table 1. *Distribution of sera containing reovirus antibodies in different age groups*

		Age group				
		6 months to 5 years	>5–10 years	>10–20 years	Over 20 years	Total
Total no. of sera tested		103	34	27	100	264
Sera with no antibody		47 (46)	10 (29)	3 (11)	13 (13)	73 (28)
No. of sera with antibody against reovirus type:	1	5 (5)	3 (9)	3 (11)	8 (8)	19 (7)
	2	3 (3)	2 (6)	1 (4)	3 (3)	9 (4)
	3	28 (27)	7 (21)	5 (19)	16 (16)	56 (21)
	1+2	3 (3)	2 (6)	4 (15)	6 (6)	15 (6)
	1+3	5 (5)	3 (9)	3 (11)	24 (24)	35 (13)
	2+3	3 (3)	1 (3)	0	3 (3)	7 (3)
	1+2+3	9 (9)	6 (18)	8 (30)	27 (27)	50 (19)
Total positive sera		56 (54)	24 (70)	24 (89)	87 (87)	191 (72)

Figures in parentheses indicate percentages.

Table 2. *Antibody pattern in different age groups according to multiplicity of infection with reoviruses*

	Age group				
	6 months to 5 years	>5–10 years	>10–20 years	Over 20 years	Total
Total no. of sera tested	103	34	27	100	264
Sera with no antibody	47 (46)	10 (29)	3 (11)	13 (13)	73 (28)
Sera with antibody to any one type of reoviruses.	36 (35)	12 (35)	9 (33)	27 (27)	84 (32)
Sera with antibody to any two types of reoviruses.	11 (11)	6 (18)	7 (26)	33 (33)	57 (22)
Sera with antibody to three types of reoviruses.	9 (9)	6 (18)	8 (30)	27 (27)	50 (19)

Figures in parentheses indicate percentages.

Multiple infections in different age groups

After rearranging the results obtained in Table 1, according to the age group and multiplicity of infection, irrespective of type or types of viruses involved, the rate of infection with any one type of reovirus did not differ markedly in different age groups, and did not decrease significantly with increasing age, suggesting thereby that infection with a single virus type might still be occuring in adults. The rate of double or triple infection increased very slowly with age.

Incidence of various types of reovirus antibodies in different age groups

Table 3 shows that, for all ages combined, the commonest reovirus infection was with type 3 and the least common was with type 2.

Reovirus type 1. The incidence of the type 1 sero-positive persons rose steadily with age up to 20 years, beyond which there was no further increase (Tables 3 and 4). The geometric mean (GM) antibody titre did not vary significantly in the different age groups ($P > 0{\cdot}05$). These findings suggest that infection with type 1 virus had been occurring at all ages up to 20 years and was not confined to childhood. The GM titres between the ages of 25 and 65 showed a slight fall ($P > 0{\cdot}05$).

Reovirus type 2. The incidence of type 2 sero-positive persons was low in all age groups (Tables 3 and 5). It showed a steady rise up to the age of 20, followed by a slow fall between 20 and 65 years. The GM antibody titres were high in the earliest age group and fell steadily throughout life. These differences were highly significant ($P < 0{\cdot}01$). The GM antibody titre against type 2 reovirus was at its lowest in the > 30–65 years age group, supporting the findings of Lerner (1963) that type 2 antibody did not persist throughout life at a high titre. It is evident that most infections with type 2 occur before the age of 10 years.

Reovirus type 3. With reovirus type 3 nearly half the children showed antibody before the age of 5 years (Tables 3 and 6). The incidence increased slowly with age up to a maximum in the > 20–25 years age group. Above 25 years there was no significant change. The GM titres in different age groups showed no significant difference ($P > 0{\cdot}05$) suggesting that infection with type 3 reovirus occurred at all ages, probably beyond 20 years.

Time of onset of infection

The more or less exact time for the onset of infection with different serotypes was obtained after further splitting the number of sera in the age group 6 months–5 years into three subgroups (Tables 4–6). The GM titre values in these subgroups were not calculated as the frequency distribution of the number of positive sera was too small in different dilutions. The figures in Tables 4–6 show that infection with all three types started between 6 months and 1 year. The numbers of positives had increased considerably for all types in the > 2–5 years age group, and it appears that a heavy shower of infection probably occurred after the age of 2 years. This might be attributed to the more frequent exposure of children over 2 to the risk of infection.

DISCUSSION

The etiologic role of reoviruses in human diseases has not been well established. They have been associated with undifferentiated febrile illness with enteritis (Rosen *et al.* 1960 *a*), common cold (Jackson, Muldoon & Cooper, 1961), hepatoencephalomyelitis (Joske *et al.* 1964), diarrhoea in children (Sabin, 1956; Ramos-Alvarez & Sabin, 1958), febrile illness with or without morbiliform rash (Lerner, Cherry, Klein & Finland, 1962), upper respiratory tract infection (Rosen *et al.* 1960 *b*). They have also been isolated from the intestinal tract of healthy children (Sabin, 1959). Probably the majority of reovirus infections run an inapparent course.

A few sero-epidemiological surveys which have been carried out suggest that the infection with reoviruses is world-wide. The incidence of neutralizing antibodies against Cincinnati 'HE' type 4 virus (now known as 'Lang' strain of

Table 3. *Incidence and geometric mean antibody titre of reoviruses in different age groups*

	Age group					
	6 months to 5 years	>5–10 years	>10–20 years	Over 20 years	Total	
Total no. of sera tested	103	34	27	100	264	
Sera with antibody against reovirus type 1	22 (21)	14 (41)	18 (67)	63 (63)	117 (44)	
G.M. titre	41	59	49	43	—	$P > 0{\cdot}05$
Sera with antibody against reovirus type 2	18 (17)	11 (32)	13 (48)	39 (39)	81 (30)	
G.M. titre	52	24	21	16	—	$P < 0{\cdot}01$
Sera with antibody against reovirus type 3	45 (44)	17 (50)	16 (59)	72 (72)	150 (57)	
G.M. titre	30	30	20	31	—	$P > 0{\cdot}05$

Figures in parentheses indicate percentages.

Table 4. *Results of haemagglutination-inhibition (HAI) test with reovirus type* 1

		Age group									
		6 months to 1 year	>1–2 years	>2–5 years	6 months to 5 years	>5–10 years	>10–20 years	>20–25 years	>25–30 years	>30–65 years	Over 20 years
Total no. of sera tested		34	23	46	103	34	27	44	24	32	100
No. of sera with titres of:	<10	30	20	31	81	20	9	16	11	10	37
	10	2	1	4	7	1	3	1	4	5	10
	20	1	—	3	4	4	3	8	—	2	10
	40	1	1	3	5	2	3	6	1	11	18
	80	—	—	—	—	3	6	7	6	3	16
	160	—	—	1	1	2	1	3	2	1	6
	320	—	—	3	3	1	2	3	—	—	3
	640	—	1	1	2	1	—	—	—	—	—
Total positive sera		4	3	15	22	14	18	28	13	22	63
		(12)	(13)	(33)	(21)	(41)	(67)	(64)	(54)	(69)	(63)
G.M. titre		—	—	—	41	59	49	54	44	36	43
								$P < 0{\cdot}05$			

Figures in parentheses indicate percentages.

Since the class frequencies in the age group 6 months to 1 year, 1–2 years, and 2–5 years are very small, statistical analysis and geometric mean titre may not be significant and reliable.

Table 5. *Results of haemagglutination-inhibition (HAI) test with reovirus type 2*

		Age group									
		6 months to 1 year	>1–2 years	>2–5 years	6 months to 5 years	>5–10 years	>10–20 years	>20–25 years	>25–30 years	>30–65 years	Over 20 years
Total no. of sera tested		34	23	46	103	34	27	44	24	32	100
No. of sera with titres of:	<10	30	21	34	85	23	14	24	15	22	61
	10	—	2	—	2	5	5	7	3	7	17
	20	1	—	—	1	2	3	8	3	3	14
	40	1	—	6	7	1	3	5	1	—	6
	80	2	—	3	5	2	2	—	2	—	2
	160	—	—	2	2	1	—	—	—	—	—
	320	—	—	1	1	1	—	—	—	—	—
	640	—	—	—	—	—	—	—	—	—	—
Total positive sera		4 (12)	2 (9)	12 (26)	18 (17)	11 (23)	13 (48)	20 (45)	9 (37)	10 (31)	39 (39)
G.M. titre		—	—	—	52	24	21	19	23	12	16
								$P > 0{\cdot}05$			

Figures in parentheses indicate percentages.

Table 6. *Results of haemagglutination-inhibition (HAI) test with reovirus type 3*

		Age group									
		6 months to 1 year	>1–2 years	>2–5 years	6 months to 5 years	>5–10 years	>10–20 years	>20–25 years	>25–30 years	>30–65 years	Over 20 years
Total no of sera tested		34	23	46	103	34	27	44	24	32	100
No. of sera with titres of:	<10	21	16	21	58	17	11	12	7	9	28
	10	4	2	6	12	5	2	3	3	5	11
	20	5	2	5	12	3	7	7	5	4	16
	40	2	1	6	9	4	3	13	6	12	31
	80	1	1	4	6	4	2	8	2	1	11
	160	1	1	4	6	1	1	—	1	—	1
	320	—	—	—	—	—	1	—	—	1	1
	640	—	—	—	—	—	—	1	—	—	1
Total positive sera		13	7	25	45	17	16	32	17	23	72
		(38)	(31)	(54)	(44)	(50)	(59)	(73)	(71)	(72)	(72)
G.M. titre		—	—	—	30	30	20	39	30	30	31
								$P > 0{\cdot}05$			

Figures in parentheses indicate percentages.

reovirus type 1) among the normal population of Cincinnati was 10 and 63 % in normal children (1–5 years) and adults (20–30 years) respectively. It was 26 % among Indian children, 1–4 years of age (Ramos-Alvarez & Sabin, 1956). In Boston and in San Juan the incidence of infection with reovirus types 1, 2 or 3 was as high as 60 % (Lerner, 1963). In England, a significant titre of HAI antibody against reovirus type 1 was observed in about 44 % of donors in the age group 11–30 years. A little higher incidence (61 %) with type 2 was noticed in the corresponding age group (Taylor-Robinson, 1963). A similar higher incidence of reovirus type 2 (87·9 %) was noted by Schmidt, Tauchnitz & Kuhn (1965). It is of interest that 80–100 % of sera collected from tropical countries including India (Taylor-Robinson, 1965) had antibodies against reovirus types 1 and 2, whereas a lower proportion of sera from temperate zones had antibodies against type 1 (47–67 %) and type 2 (67–68 %).

In the present series, children below 6 months of age were excluded. It is evident that infection with all three serotypes occurred as early as 6 months to 1 year. The presence of serum antibodies in this age group probably does not represent the maternal antibody because passively acquired antibody to reoviruses was lost by the age of six months (Lerner, 1963). A heavy shower of infection probably occurred after the age of 2 years, which might be attributed to the more frequent exposure to infection. With reovirus types 1 and 2, a maximum incidence of 67 % and 48 % respectively was attained by the age of 20 years, whereas with type 3 infection the maximum incidence of 73 % was reached by the age of 25 years. The GM antibody titres to types 1 and 3 also supported the rate of infection at different ages, and persisted almost throughout life at the same level which was attained earlier at the time of the maximum rate of infection. The reovirus type 2 antibody did not persist throughout life like the antibodies to the other two serotypes. This is borne out by a fall in GM titres to reovirus type 2 in higher age groups. The present findings show that the incidence of reovirus type 1 infection is similar to and the incidence of type 2 infection is even much lower than that in Western countries (Taylor-Robinson, 1965).

SUMMARY

Two hundred and sixty-four samples of sera obtained from normal healthy persons belonging to different age groups were examined for haemagglutination-inhibiting antibodies against reovirus types 1–3 to assess the prevalence of reovirus infection in the northern part of India.

Reovirus infection appeared as early as 6 months to 1 year of age. With reovirus types 1 and 2, the maximum incidence of 67 and 48 % respectively occurred by the age of 10–20 years, whereas with type 3 infection the maximum incidence (73 %) was reached by the age of 25 years. The incidence of reovirus type 2 infection in all the age groups was remarkably low in this series. A drop in the incidence of reovirus type 2 infection was also noticed after the age of 20 years.

The difference in geometric mean titre of antibody in different ages against reovirus type 2 was highly significant, suggesting probably that most of the infec-

tion occurred by the age of twenty years. The difference in the geometric mean value of the titres of antibody against types 1 and 3 was not significant in different age groups. The levels of antibody against types 1 and 3 in all the age groups were almost the same, suggesting that infection, specially with reovirus type 3, was occurring in all the age groups even beyond 20 years of age.

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The estimation of carrier rate from amoebic surveys

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1. INTRODUCTION

The purpose of surveys of amoebae or other organisms is the estimation of the carrier rate in a population, that is, of the proportion of persons who carry a given parasite. As a rule, the tests do not infallibly detect the presence of the organism in a known carrier. Thus simple estimation from a single examination of each member of a sample is not applicable.

Some progress has been made by Lancaster (1950), who defined a measure, the demonstrability, as the probability of a carrier being detected in one examination. His results indicated that this demonstrability varied between organisms and between persons, but remained constant over a period of time. Lancaster (1950) showed that the results of surveys could be misinterpreted if the demonstrability was assumed constant over the population of carriers. As examples he constructed mathematical models in which the demonstrability had the beta distribution. It was clear from such models that other authors had made inappropriate inferences from data collected from surveys. Lancaster (1950) reconsidered a number of surveys and showed that the estimates of carrier rates should be increased.

Here, procedures for the estimation of carrier rate are proposed, based on the assumption that the demonstrability is constant for any person between examinations, but is a random variable with some beta distribution over the population of carriers. The methods are applied to the published data of Boeck & Stiles (1923), McCoy (1936) and Andrews (1934). The expected numbers of positives in each examination and a chi-square goodness of fit statistic are calculated. Although no test of significance is available for the increase in goodness of fit due to assuming variability in the demonstrability, it is clear in many of the examples that an increase has been achieved. This indicates that better estimation of the carrier rate has been achieved by the methods proposed for the model with varying demonstrability. Finally, a simulation experiment has been performed under the models assumed, to determine the order of errors inherent in the estimation procedure.

2. METHOD OF ESTIMATION

Consider a large population with a proportion p of carriers of some parasite. A sample of N is taken from the population and each member of the sample is examined. If they are found positive they are not re-examined. Of those not found positive there may be a number of withdrawals and the remainder are re-examined and some found positive at this second examination. This process is continued until some predetermined number of examinations has been carried out.

We will consider some simpler cases which are useful preliminaries to the discussion of the solution of this general problem.

2·1. *No withdrawals*

In this section it will be assumed that each individual of the sample is examined either until an examination is positive or until he has been examined a given number of times.

2·1·1. *Equal demonstrability*

Suppose all carriers have an equal probability P of giving a positive result in any one examination. Let X_i $(i = 1, \ldots, t)$ be the number of positives in the ith examination. Then the probability of an individual being found positive in the ith examination is pPQ^{i-1}, where $Q = 1 - P$, since he must be a carrier, he must obtain negative results in the first $i-1$ examinations and then a positive result in the ith examination. The probability of an individual not being found positive in the first t examinations is $q + pQ^t$. The results of a survey and the expectations given by the model to be fitted under the assumptions of this section may be easily seen in tabular form (Table 1).

Table 1

Examination	No. examined	No. of positives	Expected no. of positives
1	N	X_1	NpP
2	$N - X_1$	X_2	$NpPQ$
...	...	...	...
t	$N - \sum_{i=1}^{t-1} X_i$	X_t	$NpPQ^{t-1}$

The number with no positives is $N - \sum_{i=1}^{t} X_i$ and its expectation is $N[1 - p(1 - Q^t)]$.

Approximations to the maximum likelihood estimates of p and P may be obtained using an iterative procedure. The formulae used for this calculation and a brief description of the method are given in the appendix. The procedure is a simple one using a computer but it would be somewhat laborious with a desk calculator.

A test of goodness of fit may be obtained by using the statistic

$$X^2 = \sum_{i=1}^{t} \frac{(X_i - NpPQ^{i-1})^2}{NpPQ^{i-1}} \tag{1}$$

as a chi-square variate with $t - 2$ degrees of freedom, where we have used the same notation for the parameters p and P, and their estimates. No contribution is obtained from the frequency of negatives since the maximum likelihood solution equates this to its expectation.

2·1·2. *Unequal demonstrability*

Now assume that each carrier in the population has a fixed probability P of giving a positive result in any examination, but that P varies between carriers. Further, assume that the frequency function of P is of the form

$$f(P) = P^{r-1}(1 - P)^{s-1}/B(r, s). \tag{2}$$

It was suggested by Bailey (1956), who considered a similar problem with a chain binomial model, that the parameters r and s be replaced by the parameters $\bar{P} = r/(r+s)$ and $Z = 1/(r+s)$. Then Table 1 may be replaced by Table 2.

Table 2

Examination	No. examined	No. of positives	Expected no. of positives
1	N	X_1	$Np\bar{P}$
2	$N - X_1$	X_2	$Np\bar{P}\bar{Q}/(1+Z)$
...	...	...	...
t	$N - \sum_{i=1}^{t-1} X_i$	X_t	$\dfrac{Np\bar{P}\bar{Q}\ldots(\bar{Q}+(t-2)Z)}{(1+Z)\ldots(1+(t-1)Z)}$

The number with no positives after t examinations is $N - \sum_{i=1}^{t} X_i$ and its expectation is

$$N\{1-p[1-\bar{Q}(\bar{Q}+Z)\ldots(\bar{Q}+(t-1)Z)/(1+Z)\ldots(1+(t-1)Z)]\}.$$

Here the likelihood equations become intractable so it is proposed that another iterative procedure be used. This procedure consists of using as an estimate of p the maximum likelihood estimate of p under the assumption that $\bar{P}$ and Z are known. This is given by the formula

$$p = \sum_{i=1}^{t} X_i / N[1-\bar{Q}(\bar{Q}+Z)\ldots(\bar{Q}+(t-1)Z)/(1+Z)\ldots(1+(t-1)Z)]. \tag{3}$$

The estimates of $\bar{P}$ and Z are obtained by minimizing the chi-square goodness of fit statistic

$$X_0^2 = \sum_{i=1}^{t} \frac{[X_i - Np\bar{P}\bar{Q}\ldots(\bar{Q}+(i-2)Z)/(1+Z)\ldots(1+(i-1)Z)]^2}{Np\bar{P}\bar{Q}\ldots(\bar{Q}+(i-2)Z)/(1+Z)\ldots(1+(i-1)Z)}. \tag{4}$$

The iterative procedure is as follows: Take $Z_1 = 0$ as a first approximation for Z, and estimate p and $\bar{P}$ by the methods of section 2·1·1. Then take these values of p, $\bar{P}$ as p_1, $\bar{P}_1$, the first approximations for p, $\bar{P}$. Now consider a grid of 25 points $(\bar{P}, Z)$, where $\bar{P}$ takes the values $\bar{P}_1$, $\bar{P}_1 \pm \delta_1$, $\bar{P}_1 \pm 2\delta_1$ and Z takes the values 0, Δ_1, $2\Delta_1$, $3\Delta_1$, $4\Delta_1$. δ_1 and Δ_1 are chosen so that the grid covers a suitable range of values of $(\bar{P}, Z)$. For every point of the grid calculate first p using the formula (3) and then X_0^2 using the formula (4), then take, as the next approximation $(p_2, \bar{P}_2, Z_2)$, those points which minimized X_0^2 among the 25 points of the grid. Now take $\delta_2 = \frac{1}{2}\delta_1$ and $\Delta_2 = \frac{1}{2}\Delta_1$, and take as the next refinement of the grid the 25 points $(\bar{P}, Z)$, where $\bar{P}$ takes the values $\bar{P}_2$, $\bar{P}_2 \pm \delta_2$, $\bar{P}_2 \pm 2\delta_2$ and Z takes the values Z_2, $Z_2 \pm \Delta_2$, $Z_2 \pm 2\Delta_2$, if $Z_2 \neq 0$, and the values 0, Δ_2, $2\Delta_2$, $3\Delta_2$, $4\Delta_2$ if $Z_2 = 0$. Now the procedure above is repeated and the next approximation, $(p_3, \bar{P}_3, Z_3)$ is obtained. The iterative procedure is continued until δ_i and Δ_i are of a specified size. Here δ_1 and Δ_1 were chosen to be 0·02 and 0·25 respectively.

2·2. *Withdrawals—treatment by life tables*

In practice in most surveys there are withdrawals resulting in incomplete data. However, if it is assumed that withdrawals are independent of the probability of

detecting a parasite in any examination, then life tables may be constructed in the way shown in Table 3.

An illustration of a constructed life table is given in Table 4 from the data of Boeck & Stiles (1923, p. 20).

Approximate estimates in the case of equal demonstrability can be obtained as in section 2·1·1 by using the life tables in the place of the data. However, the chi-square goodness of fit statistics in this case should be obtained from Table 5.

Table 3

	Data		Life table	
Examination	No. examined	No. positive	No. examined	No. positive
1	n_1	x_1	N	$X_1 = x_1 N/n_1$
2	n_2	x_2	$N - X_1$	$X_2 = x_2(N - X_1)/n_2$
...	...	...	...	...
t	n_t	x_t	$N - \sum_{i=1}^{t-1} X_i$	$X_t = x_t\left(N - \sum_{i=1}^{t-1} X_i\right)\Big/ n_t$

Table 4. *The data of Boeck & Stiles* (1923, *p.* 20) *treated by life-table methods.* Entamoeba coli

	Data		Life table	
Examination	No. examined	No. positive	No. examined	No. positive
1	8,029	1,269	100,000	15,805
2	1,441	155	84,195	9,056
3	1,050	73	75,138	5,224
4	912	44	69,915	3,373
5	791	27	66,541	2,271
6	623	13	64,270	1,341

Table 5

No. positive	Expected no. positive with constant P	Expected no. positive with varying P
x_1	$e_1 = \frac{n_1}{N} NpP$	$e_1^* = \frac{n_1}{N} Np\bar{P}$
x_2	$e_2 = \frac{n_2}{N - X_1} NpPQ$	$e_2^* = \frac{n_2}{N - X_1} Np\frac{\bar{P}\bar{Q}}{(1+Z)}$
...	...	...
x_t	$e_t = \frac{n_t}{N - \sum_{i=1}^{t-1} X_i} NpPQ^{t-1}$	$e_t^* = \frac{n_t}{N - \sum_{i=1}^{t-1} X_i} Np\bar{P}\frac{\bar{Q}\ldots(\bar{Q}+(t-2)Z)}{(1+Z)\ldots(1+(t-1)Z)}$

Then the goodness of fit statistics are

$$X^2 = \sum_{i=1}^{t} \frac{(x_i - e_i)^2}{e_i},$$

when the demonstrability is constant, and

$$X_0^2 = \sum_{i=1}^{t} \frac{(x_i - e_i^*)^2}{e_i^*}.$$

Table 6

Parasite	Constant demonstrability			Varying demonstrability				N	C
	p	P	X^2	p	$\bar{P}$	Z	X_0^2		
E. coli†	0·39	0·39	1·8	0·41	0·38	0·09	0·2	8029	3313
Endolimax nana†	0·30	0·31	25·1**	0·46	0·23	0·38	5·6	8029	3683
E. histolytica†	0·18	0·16	2·0	0·18	0·16	0	2·0	8029	1463
Endolimax williamsi†	0·10	0·35	32·6**	0·16	0·27	0·68	9·8*	8029	1278
Unencysted amoebae†	0·11	0·43	60·6**	0·18	0·36	1·23	7·4	8029	1433
Chilomastix†	0·15	0·17	21·4**	0·15	0·17	0	21·4**	8029	1231
Giardia lamblia†	0·14	0·31	51·0**	0·24	0·23	0·68	11·7**	8029	1926
Blastocystis†	0·52	0·49	161·0**	0·75	0·41	1·02	11·0*	8029	6026
Phycomycete†	0·36	0·15	1·3	0·36	0·15	0	1·3	8029	2928
E. coli‡	0·63	0·46	13·2*	0·84	0·38	0·64	1·6	505	426
Endolimax nana‡	0·33	0·23	4·8	0·34	0·22	0·02	4·8	505	173
E. histolytica‡	0·19	0·24	4·5	0·20	0·22	0·03	4·5	505	103
Giardia lamblia‡	0·17	0·35	17·1**	0·27	0·27	0·70	8·3	505	136
Blastocystis‡	0·68	0·25	1·5	0·68	0·25	0	1·5	505	343
E. histolytica§	0·49	0·34	7·7	0·58	0·30	0·16	6·0	1176	680
G. intestinalis‖	0·22	0·44	18·9**	0·32	0·36	0·79	1·7	1713	541
E. coli‖	0·33	0·44	6·3	0·38	0·40	0·23	4·2	1713	647
E. histolytica‖	0·16	0·32	7·3	0·23	0·25	0·35	4·5	1713	401

† Data of Boeck & Stiles (1923, p. 20).
‡ Data of Boeck & Stiles (1923, p. 25).
§ Data of McCoy—Hotel X (1936).
‖ Data of Andrews (1934).
* Significant at $P < 0·05$
** Significant at $P < 0·01$

for unequal demonstrability. Estimates in the latter case are obtained by using the same procedure as in section 2·1·2 to minimize X_0^2. A test of goodness of fit may be obtained by using the statistics X_0^2 as a chi-square variate with $t-3$ degrees of freedom when P is assumed to vary and X^2 as a chi-square variate with $t-2$ degrees of freedom when P is assumed constant.

3. APPLICATION TO PUBLISHED DATA

The data of Boeck & Stiles (1923, pp. 20 and 25), McCoy (1936) and Andrews (1934) have been put in appropriate form and estimates of the proportion of carriers have been made by the methods described in section 2. In all these cases, except for that of Boeck & Stiles (1923, p. 25), the data suffered from withdrawals. The data of Boeck & Stiles (1923, p. 25) were not subject to withdrawals since their surveys were of persons in institutional life. In fact here six examinations were made on each person. However, the data were in a form such that details of the number of positive examinations per person were not available and so estimation of the proportion of carriers has been done using only information on first positive examinations.

Table 6 gives the final results for all cases where estimates were made and it is clear that in some cases a considerable improvement in goodness of fit has resulted from using the model assuming variability of the demonstrability. An appropriate test for the existence of variability of the demonstrability would be a test of the hypothesis that $Z = 0$. However, no estimates of the variance of Z are available, so it is not possible to perform this test. Even so, if Z is large and X_0^2 is considerably less than X^2, it is clear that the demonstrability is not constant. Estimates of p based on the assumption that $Z = 0$ are biased down, so estimates of carrier rate based on this assumption will be, in general, too low.

Table 7. *The data of Boeck & Stiles* (1923, p. 20). Endolimax nana

No. examined	No. positive	Estimated no. positive assuming constant demonstrability		Estimated no. positive assuming variable demonstrability	
8029	855	744·1		847·6	
1441	82	103·3		94·8	
1050	43	55·2		47·8	
912	40	34·5		30·9	
791	25	21·6		21·3	
623	13	12·2		13·7	
		p	0·30	p	0·46
		P	0·31	$\bar{P}$	0·23
				Z	0·38
		X^2	25·1**	X_0^2	5·6

It is interesting to notice that estimates of $\bar{P}$, the mean demonstrability, vary quite markedly for the different parasites and surveys.

It seems worth while for the purpose of illustration to consider some cases in detail. Four have been chosen and these are set out in detail in Tables 7–10. In Tables 7 and 8, Z is reasonably large and an improvement in goodness of fit, due to fitting the model with the assumption of variability of the demonstrability, is

evident, not only in comparisons of X^2 and X_0^2 but also in a direct consideration of the tables themselves. In Table 9 the results are not so clear. There seems to be little increase in goodness of fit, but Z is not small and the increase in the estimate of p is still marked. In table 10, Z is small, there is little difference in goodness of fit and the estimates of p are not markedly different. Consideration of the two tables, 9 and 10, indicates that the appropriate measure of improvement in estimation is Z and not a comparison of X^2 and X_0^2.

Table 8. *The data of Andrews* (1934). G. intestinalis

No. examined	No. positive	Estimated no. positive assuming constant demonstrability		Estimated no. positive assuming variable demonstrability	
1713	194	164·1		195·4	
1560	71	94·5		71·7	
1093	29	38·9		29·2	
459	6	9·4		8·3	
301	4	3·5		4·0	
236	4	1·6		2·4	
		p	0·22	p	0·32
		P	0·44	$\bar{P}$	0·36
				Z	0·79
		X^2	18·9**	X_2^0	1·7

Table 9. *The data of McCoy* (1936)—*Hotel X*. E. histolytica

No. examined	No. positive	Estimated no. positive assuming constant demonstrability		Estimated no. positive assuming variable demonstrability	
1176	203	197·2		206·0	
876	123	116·2		110·0	
670	56	69·2		64·2	
558	33	41·6		40·1	
454	32	23·8		24·9	
202	8	7·5		8·9	
		p	0·49	p	0·58
		P	0·34	$\bar{P}$	0·30
				Z	0·16
		X^2	7·7	X_0^2	6·0

Table 10. *The data of Boeck & Stiles* (1923, p. 20). E. coli

No. examined	No. positive	Estimated no. positive assuming constant demonstrability		Estimated no. positive assuming variable demonstrability	
8029	1269	1234·1		1268·5	
1441	155	159·4		152·6	
1050	73	78·8		74·6	
912	44	44·6		43·7	
791	27	24·6		26·0	
623	13	12·2		14·3	
		p	0·39	p	0·41
		P	0·39	$\bar{P}$	0·38
				Z	0·09
		X^2	1·8	X_0^2	0·2

In many cases the models have enabled a very good fit to the data to be made. This suggests, at least, that the model and estimates are realistic. It is worth noting that in all cases the estimates of carrier rate are higher than those suggested in the past. A comparison of two of the estimates of the carrier rates of *E. histolytica*, 0·57 for McCoy (1936) and 0·18 for Boeck & Stiles (1923, p. 20), with the minimum estimates of the carrier rates proposed by Lancaster (1950) for these two surveys, 0·52 and 0·12 respectively, shows agreement of these estimates with his conditions.

4. SIMULATION EXPERIMENTS

The methods used in the estimation procedures do not enable us to evaluate the standard errors of the estimates, so the accuracy of the methods is uncertain. To ascertain the order of accuracy involved, two simulation experiments were performed and empirical means and standard errors of the estimates were calculated. The first experiment was performed assuming the model of equal demonstrability and in the second experiment the demonstrability was supposed to be a random variable with a specified beta distribution. In each of these cases the expected values and the variances and covariances of the number of positives at each examination were calculated. Random normal variables with zero expectations and variances and covariances equal to the calculated values were added to the calculated expected values. These values were then used as the simulated data of a survey and analysed in the same way as actual survey data. This process was repeated 20 times for each experiment and empirical means and standard errors were calculated.

In the first experiment the parameters were taken to be $N = 10{,}000$, $p = 0{\cdot}3$ and $P = 0{\cdot}3$. The empirical means of the estimates of the two parameters p and P were 0·3011 and 0·3008 respectively and the empirical standard errors per experiment of the estimates of these two parameters were 0·0069 and 0·0087 respectively. These standard errors are quite small, as could be expected from the large numbers in the simulated samples.

In the second experiment the parameters were taken to be $N = 10{,}000$, $p = 0{\cdot}3$, $\overline{P} = 0{\cdot}3$ and $Z = 0{\cdot}4$. The empirical means of the estimates of the parameters p, $\overline{P}$ and Z were 0·2943, 0·3075 and 0·3856 respectively and the empirical standard errors per experiment of the estimates of these three parameters were 0·0195, 0·0211 and 0·0593 respectively. The methods are still fairly efficient in this case, but the efficiency has been considerably reduced from that of the case of equal demonstrability. This drop in efficiency is to be expected, since there is, in this case, a large number of carriers with very low demonstrability and it is clearly difficult to separate these carriers from persons not infected.

These experiments have demonstrated that the estimates given by these methods are unbiased but that, when the demonstrability has large variation between carriers, the efficiency of the estimation procedure is lowered. Thus quite large sample numbers are necessary to ensure accurate estimation of the carrier rate when the demonstrability varies between carriers.

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APPENDIX

The likelihood function for the numbers of positives at each examination is

$$P(X_1, \ldots, X_t; N) = \frac{N!(pP)^{X_1}(pPQ)^{X_2}\ldots\{1-p(1-Q^t)\}^{N-\sum_{i=1}^{t} X_i}}{X_1!\ldots X_t!(N-\sum_{i=1}^{t} X_i)!}.$$

Now using the logarithm of this likelihood function we may use the so-called 'method of scoring' (see, for example, Rao (1966, pp. 302–309)) to obtain approximations to the maximum likelihood estimates by an iterative procedure.

Formulae are required for the values of the first and second derivatives of the logarithm of the likelihood function, $L = \log P$, with respect to p and P, and these are set out below for $p = p_j$ and $P = P_j$.

$$S_p^j = \left(\frac{\partial L}{\partial p}\right)_{p_j, P_j} = \frac{\Sigma X_i}{p_j} - \frac{(N-\Sigma X_i)(1-Q_j^t)}{1-p_j(1-Q_j^t)},$$

$$S_P^j = \left(\frac{\partial L}{\partial P}\right)_{p_j, P_j} = \frac{\Sigma X_i}{P_j} - \frac{\Sigma(i-1)X_i}{Q_j} - \frac{(N-\Sigma X_i)\,tp_jQ_j^{t-1}}{1-p_j(1-Q_j^t)}$$

$$-I_{pp}^j = \left(\frac{\partial^2 L}{\partial p^2}\right)_{p_j, P_j} = -\frac{\Sigma X_i}{p_j^2} - \frac{(N-\Sigma X_i)(1-Q_j^t)^2}{\{1-p_j(1-Q_j^t)\}^2}$$

$$-I_{pP}^j = \left(\frac{\partial^2 L}{\partial p\,\partial P}\right)_{p_j, P_j} = -\frac{(N-\Sigma X_i)tQ_j^{t-1}}{\{1-p(1-Q_j^{t-1})\}^2}$$

$$-I_{PP}^j = \left(\frac{\partial^2 L}{\partial P^2}\right)_{p_j, P_j} = -\frac{\Sigma X_i}{P_j^2} - \frac{\Sigma(i-1)X_i}{Q_j^2} + \frac{(N-\Sigma X_i)t(t-1)p_jQ_j^{t-2}}{1-p_j(1-Q_j^t)} - \frac{(N-\Sigma X_i)t^2p_j^2Q_j^{2(t-1)}}{\{1-p_j(1-Q_j^t)\}^2}$$

First estimates of p and P were taken to be $p_1 = \Sigma X_i/N$ and $P_1 = X_1/\Sigma X_i$. The iterative procedure consists of calculating δp_j and δP_j from the equations

$$S_p^j = I_{pp}^j\,\delta p_j + I_{pP}^j\,\delta P_j,$$

$$S_P^j = I_{pP}^j\,\delta p_j + I_{PP}^j\,\delta P_j,$$

and putting $P_{j+1} = P_j + \delta P_j$ and $p_{j+1} = p_j + \delta p_j$. The iteration may be stopped when a predetermined level of approximation is obtained.

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The neutralization of pox viruses

I. Evidence for antibody interference

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INTRODUCTION

Inactivated vaccinia virus can stimulate rabbits to develop neutralizing antibody and delayed hypersensitivity (McNeill, 1965, 1966), but how these factors are related to protective immunity is not known. Appleyard & Westwood (1964) have shown that the degree of protection against rabbitpox bears little relationship to levels of neutralizing antibody. Therefore either some other factor such as delayed hypersensitivity is more important, or titres of antibody measured by pock neutralization do not directly reflect its protective quality. With the latter possibility in mind the neutralization of vaccinia virus was investigated in more detail.

When a constant quantity of virus is added to a series of antibody dilutions the resulting titration profile shows two portions: (i) a persistent relatively low level of virus infectivity even in gross antibody excess, and (ii) a zone of infectivity breakthrough—the titration slope. The persistent fraction has been variously attributed to non-hereditary differences in the antigenic constitution of the particles (Dulbecco, Vogt & Strickland, 1956), dissociation on the basis of mass-law equilibria (Fazekas de St Groth & Reid, 1958), potentially infectious virus-antibody complexes (Bradish, Farley & Ferrier, 1962), non-avid antibody interfering with avid neutralizing antibody (Lafferty, 1963), and presence of virus aggregates in which some particles are protected from antibody (Wallis & Melnick, 1967). It has been generally thought that the titration slope is the result of limiting concentration of neutralizing antibody molecules whose uniformity of function is assumed.

It is the purpose of this paper to show that for pox viruses, at least, the degree of neutralization is not simply a direct relationship between virus and one type of antibody, but is the resultant effect of competing antibodies.

MATERIALS AND METHODS

The vaccinia virus, rabbit antiserum, and the methods of infectivity and neutralizing antibody titrations were as previously described (McNeill, 1965). In some experiments excess antibody was removed before assay by two cycles of centrifugation at 10,000 rev./min. for 60 min. in $3 \times \frac{1}{2}$ cm. tubes with resuspension into McIlvaine's buffer. This is referred to as washed virus. In one experiment neutralization was assayed in monkey kidney cultures in parallel with HEp 2

cell cultures. Second pass rhesus kidney was grown in 1 oz. flat bottles with Hanks's lactalbumin containing 2 % calf serum. These monolayers were inoculated for plaque assay in exactly the same way as the HEp 2 cell monolayers.

Neutralization kinetics. Stock virus (5×10^4 p.f.u./ml. in 0·004 M McIlvaine's buffer, pH 7·2, containing 20 % skim-milk (Oxoid)) and dilutions of antibody in McIlvaine's buffer were brought to a suitable temperature and mixed. The reaction was stopped at various times after mixing by making a 1/50 dilution into McIlvaine's buffer + 20 % skim-milk. When all samples were collected, residual infectivity was assayed by the standard plaque method, and expressed as the percentage of a control mixture of virus and buffer.

Serum fractionation. Antivaccinia rabbit serum was fractionated on Sephadex G-200 using a filled K 25/45 column (Pharmacia Fine Chemicals, Uppsala) and 0·01 M phosphate buffer pH 7·2 as eluent. Pools of 19 S (mercaptoethanol sensitive) and 7 S (mercaptoethanol resistant) antibody were made from fractions showing the highest neutralizing antibody activity in the two antibody elution peaks. Neither the original serum nor the fractions were heat inactivated. Sera and fractions were stored in small amounts at −20° C.

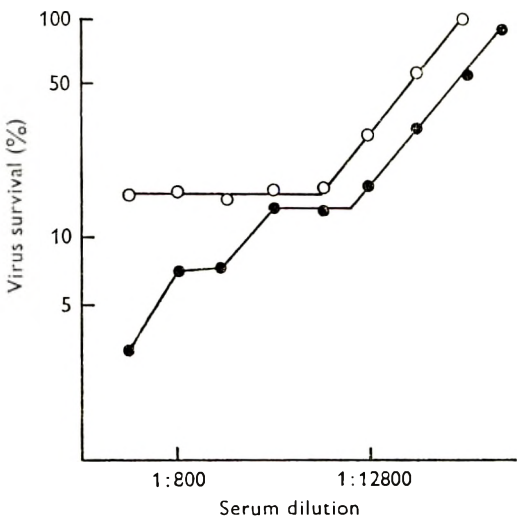

Fig. 1. Titration profile of an antiserum and the effect on this of washing the neutralized virus before assay. Filled circles, unwashed virus; open circles, washed virus.

RESULTS

Titration profile

In Fig. 1 the titration profiles are shown for a hyperimmune serum when the neutralized virus was inoculated either washed or unwashed. In the unwashed series there is a stepwise increase in percentage virus survival to a point of linear breakthrough which gives the titration slope. Washing resulted in a higher but constant level of infectivity in antibody excess, and a slight shift to the left of the titration slope. The stepwise increase in virus survival with unwashed mixtures is seen more clearly in Fig. 2, which shows the titration profiles of 7 S and 19 S

antibody. This figure shows the composite results of several experiments in which very small dilution steps were used. These dilution steps were made from starting dilutions of the antibody pools in the following series of antibody : buffer ratios.

Antibody (ml.)	0·21	0·19	0·17	...	0·05
Buffer (ml.)	0·09	0·11	0·13	...	0·25

In order to facilitate comparison of the profiles the results for each type of antibody have been superimposed without regard to the actual starting dilution of each pool. It can be seen that 7 S and 19 S antibody differ only in the steepness of the titration slope.

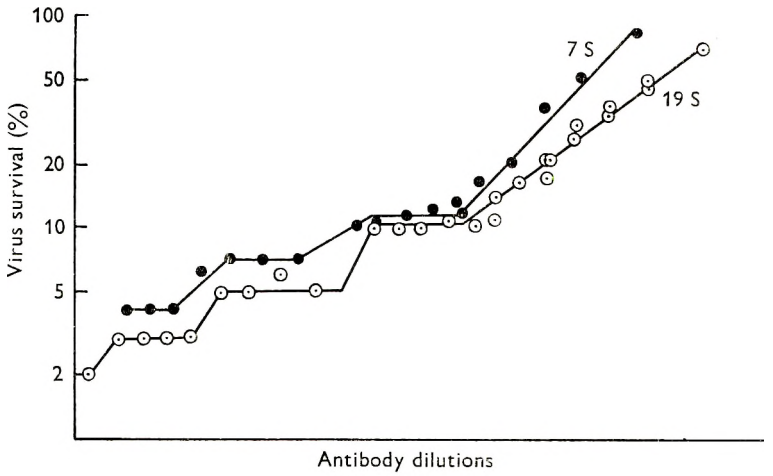

Fig. 2. Titration profiles of 7 S and 19 S antibody.

Table 1. *Percentage virus survival in a series of serum dilutions and following each of two absorptions of these dilutions*

	Percentage virus survival		
Antibody dilutions	Original dilutions	First absorption*	Second absorption*
1/4000	7	6	14
1/8000	7	8	32
1/16,000	8	11	65
1/32,000	15	35	89
1/64,000	39	51	96
50 % plaque neutralization titre*	1/80,000	1/110,000	1/52,000

* N.B. Allowance has been made for a twofold dilution of antibody with each absorption

Titration slope—antibody competition

Experiment 1. The supernatant of a virus–antibody mixture from which virus has been removed by centrifugation will neutralize fresh virus to the same percentage as the original virus. The results of the following experiment illustrate this point. A series of twofold antibody dilutions were each mixed with 300 pfu of virus and incubated for 2 hr at 37° C. One half of each mixture was kept for virus assay and

the other centrifuged for 1 hr. at 10,000 rev./min. in $3 \times \frac{1}{2}$ cm. tubes. An equal volume of virus was added to each supernate and reincubated for 2 hr. at 37° C., after which the whole process was repeated. Table 1 gives the percentage virus survival at each dilution for each of these three series of neutralizations.

Experiment 2. When additional antibody is added to a series of completed virus–antibody mixtures (*without washing the virus before the addition*) the percentage of surviving virus is not diminished. This is shown by the following experiment. Two series of twofold dilutions of high titre antiserum from 1/8000 to 1/128,000 were made and an equal volume of virus (300 pfu) added to each dilution. After 2 hr. at 37° C. an additional volume of 1/4000 antiserum was added to each tube in one series and the same volume of buffer to each tube in the other series. These mixtures were incubated for a further 2 hr. at 37° C. and the virus in each was washed by two cycles of centrifugation + resuspension in McIlvaine's buffer. This washing is essential, since excess unbound antibody can have an effect if inoculated with virus into the assay system as shown in Fig. 1. The results are shown in Table 2.

Table 2. *Percentage virus survival in a series of antibody dilutions when excess antibody is added after the first reaction*

Serum dilution	% virus survival 2nd incubation with buffer	% virus survival 2nd incubation with excess antibody
1/8000	16	17
1/16,000	32	33
1/32,000	57	45
1/64,000	79	77
1/128,000	99	89
Buffer only	100	15

Kinetics of neutralization

In these experiments antibody concentrations are expressed in arbitrary units — one unit being contained in that dilution of serum giving 50 % plaque neutralization.

7 S *and* 19 S *antibody*. The kinetic results for 3, 6 and 12 units of each type of antibody at 4° C. are shown in Fig. 3. It can be seen that 7 S neutralizes more rapidly than 19 S at 'equivalent' concentration; that with both types there is a change in rate during the course of neutralization; and that the increase in rate with increasing concentration is greater for 7 S than it is for 19 S. Figure 4 shows that increasing the temperature also increases the rate of neutralization by 7 S more than that by 19 S.

Different assay systems. Figure 5 shows the kinetic slopes of virus neutralization with 12 units of antibody at 37° C. when the same reaction mixtures were assayed in monkey kidney (M.K.) and HEp 2 cells. It is clear that the rate of neutralization is apparently much slower when assayed in M.K. Other experiments showed that the level of persistent infectivity in 100 units of antibody after 4 hr. at 37° C. was much higher in M.K. than in HEp 2 (30 % compared with 4 %). The mean

50 % plaque neutralization titre for the serum was 1/20,000 in HEp 2 and 1/5000 in M.K. The virus control counts showed that the HEp 2 cells were three times as sensitive to virus as the M.K.

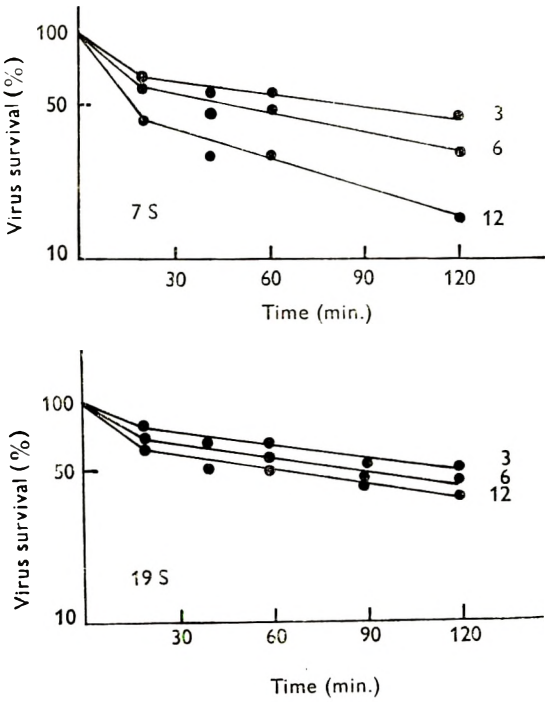

Fig. 3

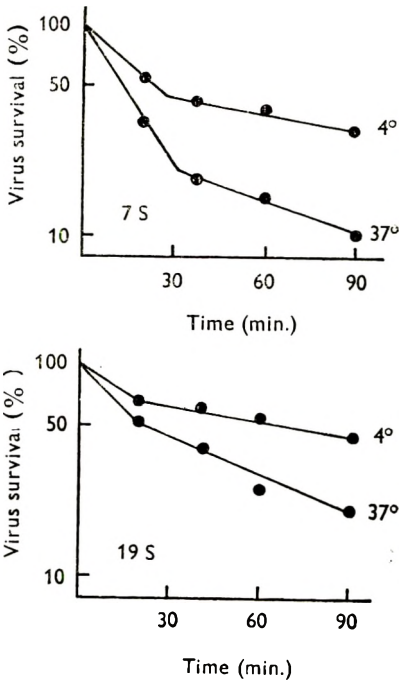

Fig. 4

Fig. 3. The kinetics of neutralization of vaccinia with 7 S and 19 S antibody at 4° C. Effect of antibody concentration (3, 6 and 12 units).

Fig. 4. Kinetics of neutralization of vaccinia with 7 S and 19 S antibody. Effect of temperature (4 and 37° C.).

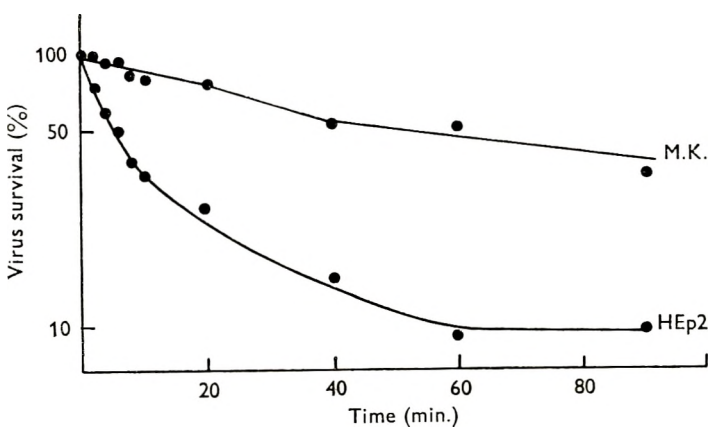

Fig. 5. Kinetics of neutralization. The effect of assaying the same reaction mixtures in HEp 2 cells and monkey kidney.

DISCUSSION

The titration slope

Experiment 1 shows that antibody is present in excess at dilutions corresponding to the titration slope. Experiment 2 shows that a partially neutralized virus suspension on the titration slope cannot be further neutralized by the addition of more antibody. The only explanation of these findings is that such unneutralized virus has become protected from neutralizing antibody. The percentage virus survival at any serum dilution must be the resultant of virus–antibody reactions having opposite effects. However, since both types of antibody would be present in the same proportion at all serum dilutions it is difficult to explain the sharp transition in percentage virus survival which gives the titration slope. It could be postulated either (*a*) that neutralizing (*N*) antibody is much less avid than interfering (*I*) antibody, or (*b*) that a third type of antibody is involved—anti-interfering (*AI*)—which when combined with sites adjacent to an *I* site can block the attachment of *I* antibody thus leaving the *N* site open for *N* antibody. If the *AI* antibody reached limiting dilution first in a series of serum dilutions, further dilution would result in virus neutralization becoming progressively more dependent upon straightforward competition between *N* and *I* antibody, thus giving the titration slope. The steepness of the slope would depend upon the $(N + AI)/I$ ratio at the appropriate dilutions both in terms of relative concentrations and quality of the various antibody molecules.

Such a model is necessarily speculative but is at least consistent with the observations that when 7 S and 19 S antibody are compared the increase in neutralization rate with increasing concentration of antibody (Fig. 3) and increasing temperature of reaction (Fig. 4) is less for 19 S than 7 S. This effect could be simply explained by the interference hypothesis on the basis of molecular size if the 19 S *AI* molecules not only block the attachment of *I* antibody, but being large molecules also can interfere with the attachment of *N* antibody.

The persistent fraction

A consequence of the interference hypothesis is that even at high antibody concentrations there will be a proportion of virus particles protected by *I* antibody. This would contribute to the persistent fraction for which various explanations have been listed in the introduction. Wallis & Melnick (1967) have proposed that the persistent fraction for several viruses, including vaccinia, is due to the presence of virus aggregates. This may account for it in part, but cannot account for it entirely, since that would be inconsistent with several features reported by Lafferty (1963): (*a*) addition of excess antibody to *washed* virus in the persistent fraction further decreases infectivity, (*b*) the same virus preparations against different antisera can show markedly different persistent levels, and (*c*) the persistent fraction can be greatly influenced by the assay system. If part of the persistent fraction depended upon the resultant effect of competing antibodies on and around critical neutralization sites it would be expected to vary with different antisera. The cell-dependent aspect of the persistent fraction is particularly

interesting, and Lafferty (1963) suggested that it was due to antibody recognized as neutralizing in one system but not in another, such antibody even protecting the virus from antibody having a neutralizing effect for the second system.

A simpler explanation using the interference hypothesis would be that to ensure neutralization some cells require more virus *N* sites to be inactivated than other cells. The greater the number of *N* sites which have to be neutralized the greater will be the opportunity for *I* antibody to protect the virus and one manifestation of this will be a higher persistent fraction. Therefore it could be predicted that when one assay system required neutralization of more *N* sites than another it will show apparently slower neutralization kinetics, a higher persistent fraction and a lower 50 % plaque reduction neutralization titre for a serum. The results of parallel assays of virus–antibody mixtures in HEp 2 and M.K. (Fig. 5) are consistent with these predictions.

The phenomenon of antibody interference is obviously relevant to the value of an antibody response in protection against virus infection, since it would be expected that the protective quality of the response would depend on the proportions and relative avidity of the neutralizing and interfering antibodies as well as on the absolute concentration of neutralizing antibody. This phenomenon may at least partly explain why some authors have observed little correlation between levels of neutralizing antibody and the degree of protection against infection.

SUMMARY

Evidence was presented to support a hypothesis that competition between antibody molecules can be an important factor in vaccinia virus neutralization.

It has been shown that there is little difference in the virus neutralizing properties of 7 S and 19 S antibody and it has been further postulated that in the context of protective immunity the proportions of antibodies having neutralizing and interfering effects is likely to be more important than the absolute concentration of neutralizing antibody or the type of immunoglobulin in which it is present.

I wish to thank Mr Mervyn Killen for excellent technical assistance. I would also like to thank Prof. K. B. Fraser for some very helpful discussions during the preparation of the manuscript.

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The neutralization of pox viruses

II. Relationships between vaccinia, rabbitpox, cowpox and ectromelia

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INTRODUCTION

The serological relationships between members of the vaccinia subgroup of pox viruses have been the object of several investigations. Downie & Macdonald (1950) and Macdonald & Downie (1950), using complement fixation with soluble antigens and rabbit antisera, showed that the degree of fixation tended to be greatest in homologous systems but did not clearly differentiate the viruses. Complement-fixation inhibition tests using fowl antisera showed that cowpox and ectromelia were more closely related to each other than either was to variola or vaccinia. Similar conclusions were reached by Gispen (1955) using immunodiffusion. Very little information is available concerning the cross-relationships of these viruses in neutralization tests. Downie & McCarthy (1950), using pock-neutralization, were able to demonstrate differences by cross-neutralization and after cross-absorptions, but these experiments did not reveal any obvious quantitative pattern, and, as the authors point out, interpretation of the cross-absorption experiments was difficult since the antisera were not of comparable potency. Appleyard & Westwood (1964), using a 50% pock neutralization end-point, have shown that the titre of an antivaccinia serum can be quite different not only when titrated against rabbitpox virus, but also when titrated against different strains of vaccinia. The greater precision now available with plaque methods makes it reasonable to re-investigate these viruses by cross-neutralization in the hope that more definite relationships can be established. This paper is concerned with these relationships determined in the HEp 2 cell-assay system.

MATERIALS AND METHODS

Virus strains. The vaccinia was as described previously (McNeill, 1965). Rabbitpox Utrecht strain, cowpox Brighton strain and ectromelia Mill Hill strain were supplied by Dr C. R. Madeley. Each virus was passed several times in HEp2 cells and suspensions were prepared for neutralization tests after partial purification by differential centrifugation and resuspension with ultrasonic vibration. Virus was diluted to a concentration of 10^5 pfu/ml. in McIlvaine's buffer 0·004 M pH 7·2, containing 20% skim-milk (Oxoid). These stock suspensions were stored in small volumes at $-70°$ C. For kinetic studies the stock suspension was used at a 1/2 dilution and for neutralizing antibody titrations at 1/100, each dilution being made in 0·004 M McIlvaine's buffer containing 20% skim-milk.

Virus titrations and titrations of neutralizing antibody for each virus were performed by the method previously described for vaccinia (McNeill, 1965) with two modifications—(i) the antiserum in the overlay medium was homologous for the virus being titrated, and (ii) on account of their minuteness ectromelia plaques were given an extra day to develop.

Kinetic studies were performed by the dilution method described in the previous paper (McNeill, 1968).

Antisera

The preparation of antivaccinia serum has been described previously (McNeill, 1965).

Anti-rabbitpox. Four rabbits were each inoculated with 50 pfu virus intraperitoneally (I.P.), and the following day with 1 ml. high titre anti-rabbitpox serum intravenously (serum supplied by Dr G. Appleyard). Three weeks later each rabbit was given 10^6 pfu I.P. Following this inoculation two of the rabbits developed a fatal infection. The two survivors received two further I.P. doses of 10^7 pfu at 2-week intervals after this. The animals were bled out 1 week after the last inoculation and their sera pooled.

Anti-cowpox. Two rabbits were each given 10^6 pfu virus I.P. and this was followed 1 month later by a booster inoculation of 10^7 pfu. The animals were bled out 2 weeks after the second inoculation and their sera pooled.

Anti-ectromelia. Two rabbits were each given a course of five I.P. inoculations of 1 ml. virus suspension containing 10^8 pfu/ml. at 2-weekly intervals. They were bled out 2 weeks after the last inoculation and their sera pooled. As ectromelia does not infect rabbits the inoculated material consisted of ground-up infected chorioallantoic membranes which was clarified by light centrifugation. No attempt was made to purify the virus any further, since it was hoped that the soluble antigens present in the extract would give an antigenic stimulus similar to that given by the other viruses which produce soluble antigens during the course of infection.

All sera were sterilized by Seitz filtration and stored at $-20°$ C.

Serum fractionation. Pools of 7 S and 19 S antibody were prepared from each antiserum by the method described in the previous paper (McNeill, 1968).

RESULTS

Antibody titrations

50 % plaque reduction titres

7 S and 19 S antibody fractions for each virus were titrated against each virus and the results are given in Table 1. Each value is the average of at least four separate determinations.

It can be seen that: (i) vaccinia and rabbitpox give very close cross-titration values, (ii) the titre obtained when cowpox antibody is titrated against vaccinia or rabbitpox is approximately one half of that when cowpox virus is used, (iii) when vaccinia, rabbitpox or cowpox antibody is titrated against ectromelia the

titre is approximately one tenth that for the homologous virus, (iv) the titre of ectromelia antibody is approximately doubled when titrated against vaccinia or rabbitpox compared with ectromelia, (v) these relationships hold for both 7 S and 19 S antibody.

Table 1. *Cross-neutralizations with 7 S and 19 S antibody*

	Antibody							
	Vaccinia		Rabbitpox		Cowpox		Ectromelia	
Virus	7 S	19 S	7 S	19 S	7 S	19 S	7 S	19 S
Vaccinia	500	76	3550	90	125	38	280	22
Rabbitpox	650	95	3620	118	115	23	236	15
Cowpox	220	26	2150	94	384	56	340	28
Ectromelia	44	5	385	9	31	7	140	8

Table 2. *Slopes of titration lines in cross-neutralization tests with 7 S and 19 S antibody*

	Antibody							
	Vaccinia		Rabbitpox		Cowpox		Ectromelia	
Virus	7 S	19 S	7 S	19 S	7 S	19 S	7 S	19 S
Vaccinia	60°	40°	43°	49°	52°	43°	42°	43°
Rabbitpox	44°	44°	57°	42°	46°	43°	44°	41°
Cowpox	54°	45°	48°	46°	57°	43°	46°	48°
Ectromelia	37°	35°	41°	40°	40°	38°	60°	40°

Slope of titration lines

The slopes of the line relating serum dilution to percent virus survival are given in Table 2. The slopes are measured as the angle between the titration line and the abscissa using points between 20 and 70 % virus survival. It can be seen (i) that the slope for 7 S antibody in homologous systems is steeper than in heterologous systems, and (ii) that with 19 S antibody there is no consistent difference between homologous and heterologous systems.

Virus survival in excess antibody

The persistent virus infectivity in the region of antibody concentration just before infectivity breakthrough was measured for each virus against 7 S and 19 S antibody for each using fine dilution steps. It was found that each 7 S antibody preparation neutralized each virus to the same general level with the exception of ectromelia virus, which showed a slightly higher level of survival than the other viruses with all 7 S antibodies including its own. With 19 S antibody the outstanding feature was that ectromelia gave considerably higher levels of surviving virus with all antibody including its own. This is shown in Fig. 1, where the levels have been plotted to allow for the quantitative differences given in Table 1. With the additional exception of rabbitpox, which gave a higher persistent

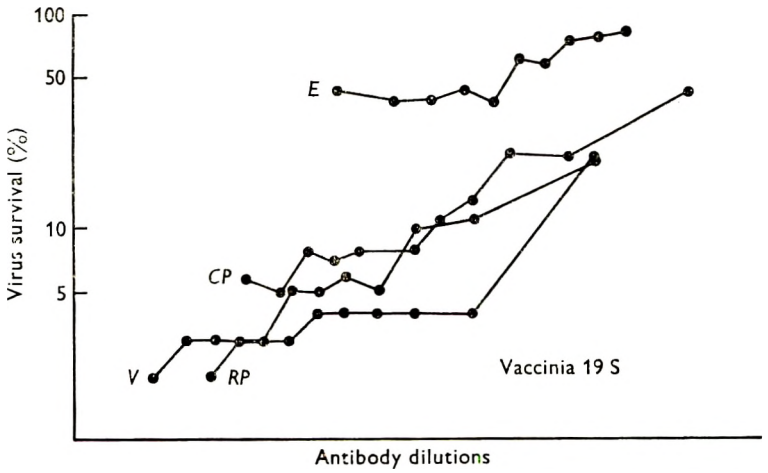

Fig. 1. Relationship between percentage virus survival and concentration of vaccinia 19 S antibody for ectromelia (*E*), cowpox (*CP*), vaccinia (*V*), and rabbitpox (*RP*). Results are plotted to take account of the titration differences shown in Table 1.

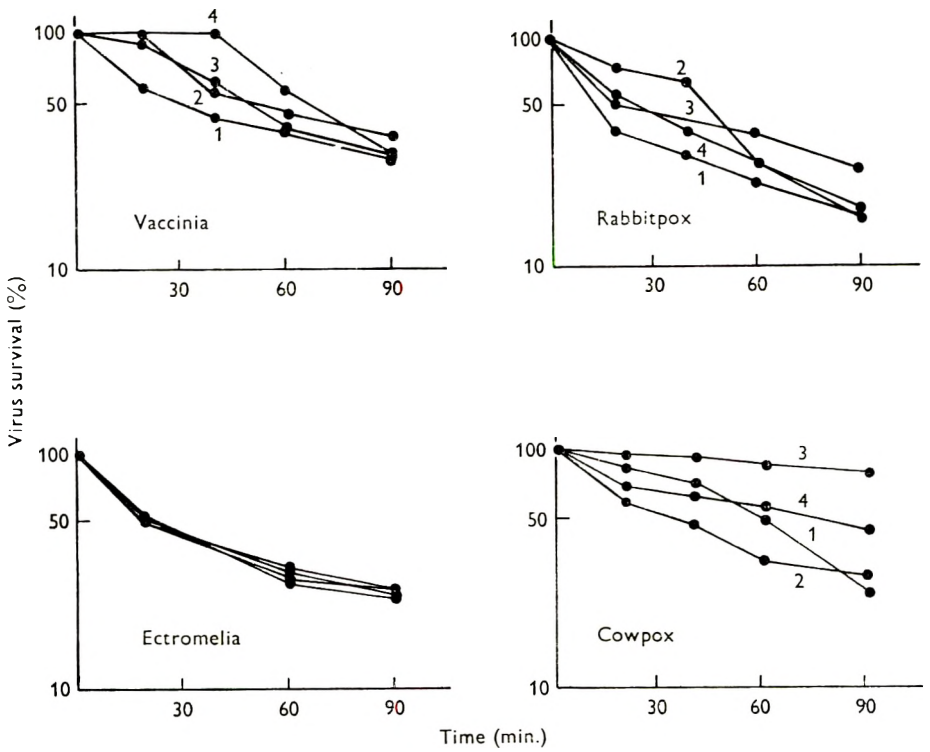

Fig. 2. Kinetics of neutralization of vaccinia, rabbitpox, ectromelia and cowpox viruses by 12 units of vaccinia 7 S (1), rabbitpox 7 S (2), cowpox 7 S (3), and ectromelia 7 S (4) at 4° C.

fraction in excess cowpox 19 S antibody, all the other virus-antibody systems gave much the same levels of persistent infectivity.

Kinetics of neutralization

Figure 2 shows the kinetic slopes obtained in reactions with all 7 S antibodies. In each experiment the reaction temperature was 4° C. and antibody was used at a concentration of 12 units with reference to the titre of the antiserum with the particular virus, i.e. 12 units of vaccinia 7 S against vaccinia represented a 1/40 dilution, whereas 12 units of this serum against ectromelia represented a dilution

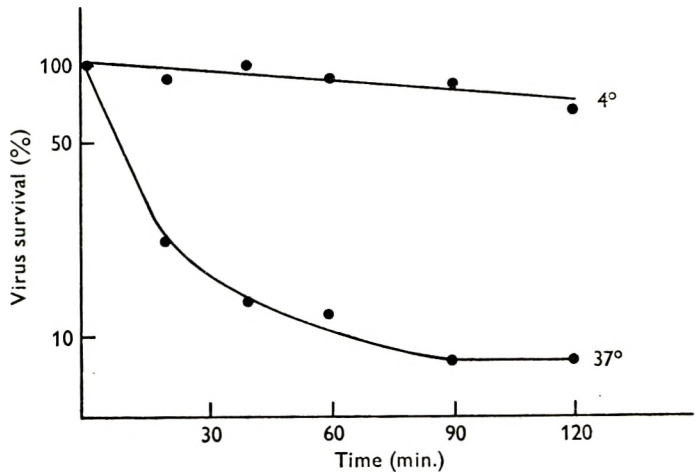

Fig. 3. Kinetics of cowpox neutralization by 12 units of anticowpox 7 S antibody at 4° C. and 37° C.

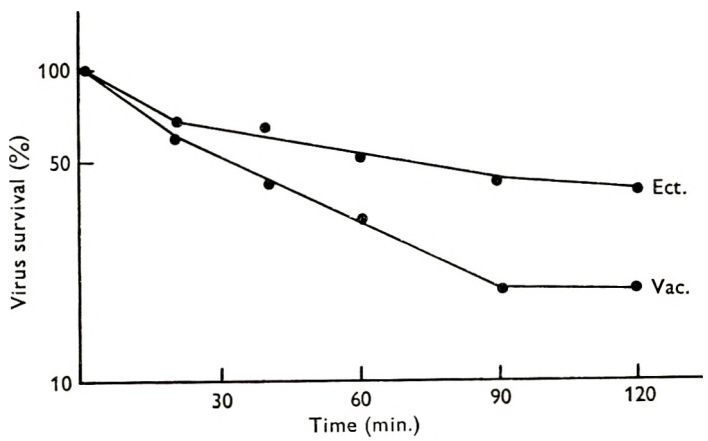

Fig. 4. Kinetics of neutralization of ectromelia and vaccinia viruses by 4 units of antiectromelia 19 S at 37° C.

of 1/4. It would be expected that the rate of neutralization be the same at equivalent concentrations of all antisera. However, this was only seen with ectromelia and the results with the other viruses were more complicated, each of these

showing distinct shoulders on the kinetic curves with some antisera. It is noteworthy that cowpox was neutralized very poorly by its own antibody at 4° C., although when the reaction was carried out at 37° C. neutralization proceeded normally (Fig. 3).

In the light of the observation that ectromelia showed a high persistent fraction in excess 19 S antibody, kinetic studies were undertaken to determine if this was due to a prolonged initial delay in the neutralization reaction or to a normal initial reaction leading to a high persistent fraction. Figure 4 shows the kinetics of ectromelia and vaccinia neutralization by four units of ectromelia 19 S antibody, and this demonstrates that the latter alternative is correct.

DISCUSSION

These results have confirmed the very close immunological relationship of the viruses concerned and in addition have revealed quite precise relationships in cross-titration values. Rabbitpox and vaccinia were indistinguishable on cross-titrations, whereas cowpox and to a greater extent ectromelia are clearly separable from each other and from rabbitpox and vaccinia. It must be emphasized that these relationships can only apply to the particular strains studied using the HEp 2 cell assay system. Appleyard & Westwood (1964) found clearly distinguishable cross-titration differences between rabbitpox and vaccinia using the chorioallantoic membrane as assay system, so it is obvious that no general interpretation of these differences is justified on the basis of results from a single assay system with single strains of virus. The results of vaccinia neutralization in HEp 2 and monkey kidney described in the previous paper emphasize the same point. The reduction in titration slope noted by Appleyard & Westwood (1964) when vaccinia and rabbitpox viruses were titrated against heterologous antisera has been shown to apply to all four viruses in heterologous systems with 7 S antibody only. On the basis of the hypothesis of antibody competition proposed in the previous paper (McNeill, 1968) a reduction in titration slope indicates that the resultant effect of competition is proceeding in favour of neutralization. In the rabbitpox-vaccinia systems, where the titration end-points and presumably the number of antibody molecules involved are the same, the reduction in slope in heterologous systems is most simply explained by assuming that the antigenic sites for neutralization (N) of the two viruses are identical, and that the difference lies in the avidity of interfering (I) antibodies. This difference must be slight, and could be due to minor variations in the structure of I antigens or to the same I antigen being presented at the virus surface in a slightly different manner. The cross-neutralization kinetic results shown in Fig. 2 are also consistent with the conclusion that the viruses possess identical N antigens. The most straightforward results were with ectromelia, which gave identical kinetics with 12 units of all four antisera. A marked initial delay in the neutralization of vaccinia was seen with heterologous antibody. However, the results with rabbitpox and cowpox clearly show that this delay is not confined to heterologous systems, and neither is it a consistent feature of all reactions with a particular virus. It could be due to

dissociation of *N* antibody on dilution of the virus-antibody mixtures. Lafferty (1963) has shown that this instability on dilution is very marked with monovalent antibody fragments and has proposed that stabilization occurs when the antibody molecule has made a bivalent attachment to the virus.

It has been shown that ectromelia virus gives a high persistent fraction with the 19 S antibody of all four viruses (Fig. 1), and that this is not due to any delay in the initial rate of neutralization (Fig. 5). This effect is therefore a property of 19 S antibody reacting with ectromelia virus in particular and could indicate that the *N* sites of ectromelia are relatively inaccessible and therefore attachment of *N* antibody is more easily blocked by interfering antibody, especially when it is of macroglobulin type.

SUMMARY

Plaque neutralization on HEp 2 cells was used to determine the relationship between vaccinia, rabbitpox, cowpox and ectromelia using both 7 S and 19 S antibody. It was shown that for the strains of virus used vaccinia and rabbitpox were very similar but cowpox and particularly ectromelia were clearly distinguishable. The cross-titration relationships were the same for 7 S and 19 S antibody.

The titration slopes and kinetics of cross-neutralizations were interpreted on the basis of a hypothesis of antibody competition put forward in the preceding paper. It was tentatively concluded that these four viruses share a common critical antigen for neutralization and that differences in the structure or presentation of neighbouring antigens could give rise to some of the observed effects.

I wish to thank Mr Mervyn Killen for excellent technical assistance. I also wish to thank Prof. K. B. Fraser for some very helpful discussions during the preparation of the manuscript.

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The inactivation of poliovirus in aerosols

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INTRODUCTION

Many viruses are far less stable in aerosols than in aqueous solutions. For poliovirus the kinetics of the decay in air have been described by Hemmes, Winkler & Kool (1960, 1962) and Harper (1961, 1963). The mechanism, however, remained obscure.

The only pertinent theory on virus death in air is that of Webb, Bather & Hodges (1963). They developed from indirect evidence the concept that the primary event, causing the decay of bacteria and viruses in air, is the withdrawal of stabilizing water molecules from the nucleic acid of the organism. The nucleic acid structure then collapses and irreversible secondary reactions may occur.

We have tried to obtain direct evidence on the mechanism of the decay of poliovirus in aerosols by studying among other things the biological activity of infectious RNA extracted from aerosolized poliovirus.

MATERIALS AND METHODS

Virus

Poliovirus type 1, strain LSc2ab, was grown and titrated by the plaque method on monolayers of human amnion cells, line U (Doorschodt, 1961). The growth medium consisted of lactalbumin hydrolysate 0·5 % and calf serum 5 % in Hanks's balanced salt solution. The virus was concentrated 20–200 times by means of ultracentrifugation. Before spraying, the virus was diluted 1/2 in Dulbecco's phosphate buffered saline (PBS) containing 1 % peptone.

Infectious RNA (iRNA) was extracted, after concentration by ultracentrifugation, from the collection fluids with the conventional phenol method (Alexander, Koch, Mountain & van Damme, 1958). The extracts contained 1·5 M-NaCl and had a pH of 8. With these extracts human amnion cell monolayers were infected and processed for plaque formation.

The aerosol equipment

Virus suspensions of 1 ml. were sprayed directly in 4 sec. with an atomizer of the type FK 8 by a stream of pure nitrogen. The diameter of the produced droplets ranged from 3 to 5 μ with 10 % larger than 10 μ. The mean 'physical fall-out' corresponded to a K-value (see below) of 0·1 per hr. and was neglected

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in the calculations of biological decay. The aerosols were generated in air at 20° C. in an experimental room of 2000 l. schematically represented in Fig. 1.

Air samples were taken with conventional capillary impingers (Rosebury and others, 1947) at a rate of 10 l./min. Sampling time was 5 min.; the first sample started 1 sec. after the end of the spraying period. As a collection fluid 10 ml. PBS with 1 % peptone (Difco Bacto-peptone) and 0·1 % antifoam AF (Dow Corning Corp. U.S.A.) was used.

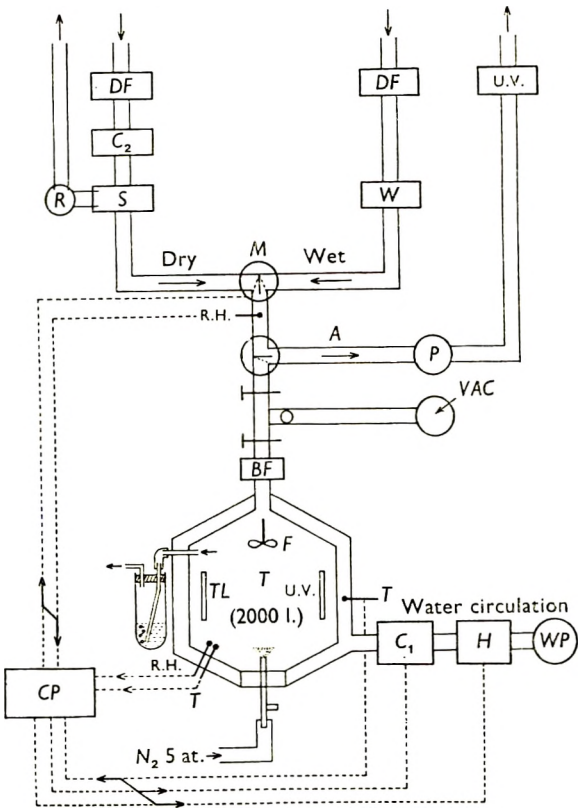

Fig. 1. Scheme of apparatus for producing, storing and sampling of aerosols. Tank *T* has a double wall, in which thermostatically heated water circulates to maintain a temperature of 20° C. (pumping by *WP*, heating by *H*, cooling by C_1). By means of pump *P* fresh air (filtered by dustfilters *DF*) is dried over silicagel *S* (to regenerate with *R*) or humidified over a water surface *W*. These two streams are mixed in the mixing valve *M*, giving an air stream *A* of the desired relative humidity (R.H.). After evacuation, the tank can be filled with sterile air of the required R.H. through the bacteria retaining filter *BF*. The continuous air stream *A* also replaces the air withdrawn during sampling. The composition of the air in the tank is kept uniform by a fan *F* and can be sterilized by ultraviolet radiation. Temperature and R.H. are recorded and regulated automatically by the control panel *CP*. The apparatus was designed by Dr K. C. Strasters and Prof. Dr K. C. Winker and built by 'Lucht- en Droogtechniek N.V.' in Rotterdam.

RESULTS

Influence of the relative humidity on the decay rate

An organism sprayed in air is exposed to the stress of spraying, to the quick evaporation till the droplets are in equilibrium with the ambient air and to the decay in the stable aerosol during storage. The mechanisms of inactivation during these three phases might differ. For this reason the inactivation during the first minutes (till the end of the first sample) is treated separately from the loss during storage of the stable aerosol.

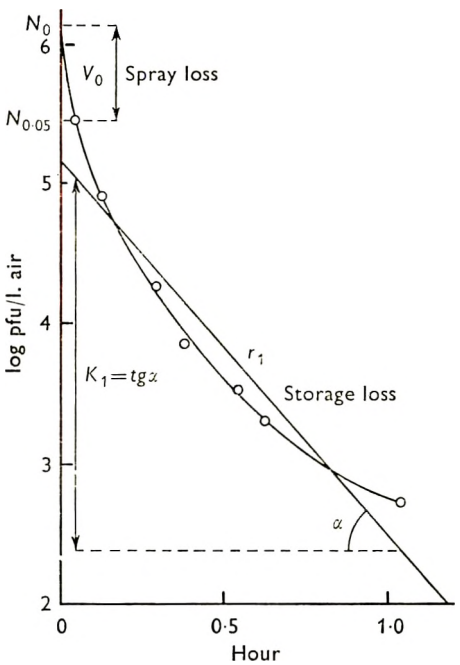

Fig. 2. Inactivation of poliovirus in air with 45% relative humidity. Symbols as in Fig. 3; r_1 = regression line over the first hour.

Figure 2 shows the outcome of a typical experiment and demonstrates the symbols used in this report. The spray-loss V_0 is defined as the logarithm of the quotient of the calculated virus concentration in the aerosol (no decay) and the actual concentration as deduced from the titre of the first sample. The rate of loss during storage, K, is recorded as the logarithm of the concentration reduction factor over an hour.

K-values were calculated with the method of least squares from the experimental titres. As the decay rate is slowing down with time, K is dependent on time. Nevertheless, the K-values of experiments with the same sampling schedule can be used for the comparison of inactivation rates under different conditions.

The influence of relative humidity (R.H.) is summarized in Fig. 3, in which spray-loss V_0 and storage-loss-rate K are plotted against R.H.

The spray loss was large at low R.H. and decreased with rising humidity, being

minimal above 55% R.H. The storage-loss-rate was low below 35% and above 70% R.H. but high between 40 and 55% R.H. This range of maximal storage-loss-rate coincides with the range of decrease of the spray-loss.

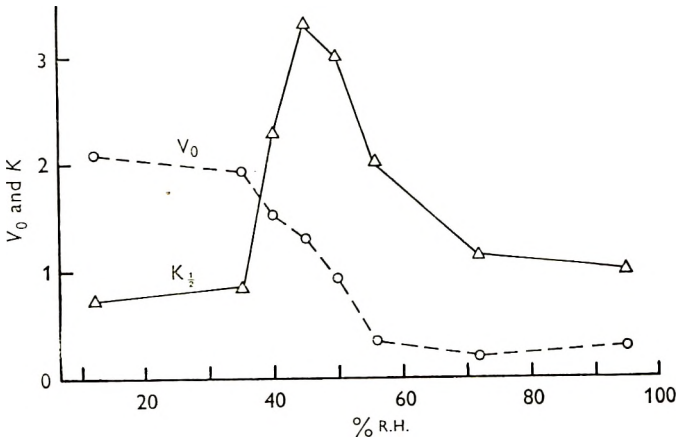

Fig. 3. Inactivation of poliovirus in air in relation to the relative humidity (R.H.). $V_0 = \log N_0 - \log N_{0\cdot04}$ with N_0 = calculated titre (no decay) and N_t = actual titre after t hr. $K_t = (\log N_{0\cdot04} - \log N_t) \times 1/(t - 0\cdot04)$.

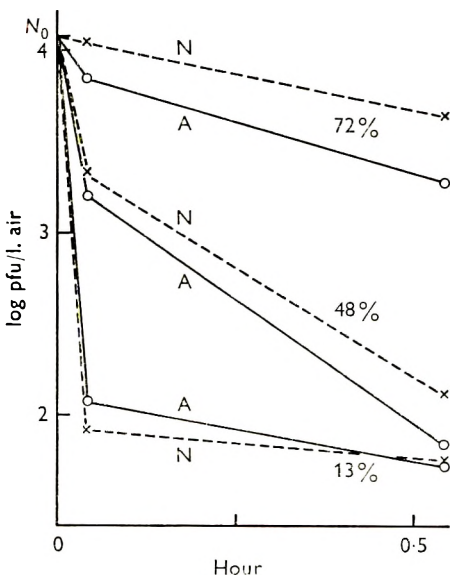

Fig. 4

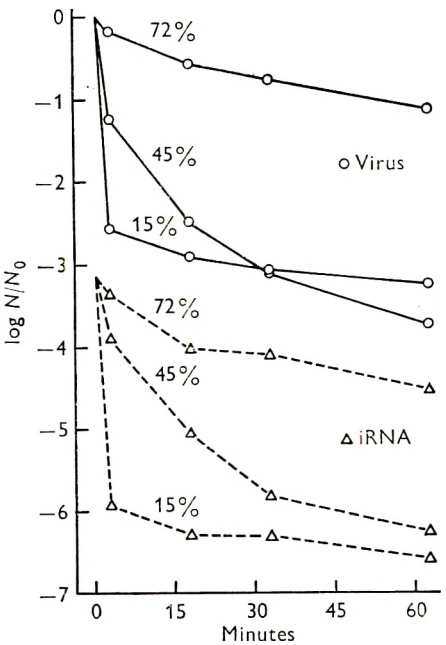

Fig. 5

Fig. 4. Inactivation of poliovirus in nitrogen (N) and in air (A) at 13%, 48% and 72% R.H.; pfu = plaque-forming units.

Fig. 5. Comparison of the decay of poliovirus in air with the inactivation of the RNA in the virus particles. The mean titres of four experiments are plotted against time.

Influence of oxygen

To determine the role of oxidation, poliovirus was nebulized and stored in a nitrogen atmosphere. In Fig. 4 the titres are plotted against time for corresponding experiments in nitrogen (0·1 % oxygen) and in air.

No significant difference was noted between the inactivation of poliovirus in the two gases. Therefore, oxygen is not an important factor in the decay of the virus.

Inactivation of the RNA in the poliovirus particle

Batches of virus containing 3×10^9 pfu/ml. were sprayed in air of low (15–18 %), moderate (45 %) or high (71–72 %) R.H. All aerosols were sampled from 0 to 5, 15 to 20, 30 to 35 and 60 to 65 min. after nebulization. The impinger fluids were titrated for whole virus and for infectious RNA (iRNA).

Table 1 and Fig. 5 represent the results. In Table 1 the 'nucleic acid extraction and infection efficiency' (N.E.) is calculated as the quotient of the titres of iRNA and virus. The titres plotted in Fig. 5 are the mean titres of the four experiments given in Table 1.

Table 1. *Inactivation in air of poliovirus and of the RNA within the virus particles*

	15–18 % R.H.			45 % R.H.			71–72 % R.H.		
	$K_1(V)$*	$K_1(R)$†	N.E.‡	$K_1(V)$	$K_1(R)$	N.E.	$K_1(V)$	$K_1(R)$	N.E.
1	0·7	—	—	1·9	3·3	5×10^{-4}	0·7	1·4	4×10^{-4}
2	0·8	0·7	16×10^{-4}	2·1	2·2	15×10^{-4}	1·0	1·1	15×10^{-4}
3	0·5	0·5	7×10^{-4}	2·5	1·7	6×10^{-4}	1·1	0·7	4×10^{-4}
4	0·5	0·7	9×10^{-4}	2·7	2·3	7×10^{-4}	0·7	0·8	9×10^{-4}
Mean	0·62	0·62	$9{\cdot}9 \times 10^{-4}$§	2·29	2·37	$8{\cdot}6 \times 10^{-4}$	0·90	1·02	$7{\cdot}8 \times 10^{-4}$

* $K_1 = -\Delta$ log titre per hour calculated with the method of least squares from the four observed titres of the first hour. $K_1(V) = K_1$ of the whole virus.

† $K_1(R) = K_1$ of the infectious RNA (iRNA).

‡ N.E. = 'nucleic acid extraction and infection efficiency' = titre iRNA : titre virus; with not-sprayed virus the N.E. amounts to $5{\cdot}9 \times 10^{-4} \pm 0{\cdot}6 \times 10^{-4}$.

§ The high mean values of N.E. are due to Expt. 2.

Table 1 and Fig. 5 show a close parallelism between the inactivation of the whole virus and the iRNA. Also the N.E. does not differ from that of unaerosolized virus.

Koch (1960) studied the thermal inactivation of poliovirus in fluid media and described that after inactivation at 54° C. many apparently uninfective particles were still infective in hypertonic medium. Hence we titrated our impinger fluids by infecting the monolayer cells in hypertonic as well as in isotonic medium. No significant difference was observed in our system, indicating that few particles with a missing or damaged protein coat and intact RNA were present.

The results obtained suggest that the virus decay is due to inactivation of the RNA.

Influence of preincubation with L-*cystine*

Preincubation with L-cystine protects poliovirus against inactivation at 50° C. (Pohjanpelto, 1958). The amino acid is thought to react with SH-groups of the virus protein, thus stabilizing its structure. Consequently we investigated the decay of poliovirus in air using virus incubated with 50 (occasionally 500) μg L-cystine/ml. for 15 hr. at 37° C. and pH 8.

For these experiments a batch of poliovirus was grown in Hanks's balanced salt solution in the absence of lactalbumin hydrolysate and serum (which contain L-cystine): 'virus-H'. This virus indeed responded to stabilization against heating at 50° C. by L-cystine approximately as reported by Pohjanpelto. Before and after incubation with the amino acid the inactivation factor was $10^4\times$ and $2\times$ respectively after 5 min. at 50° C.

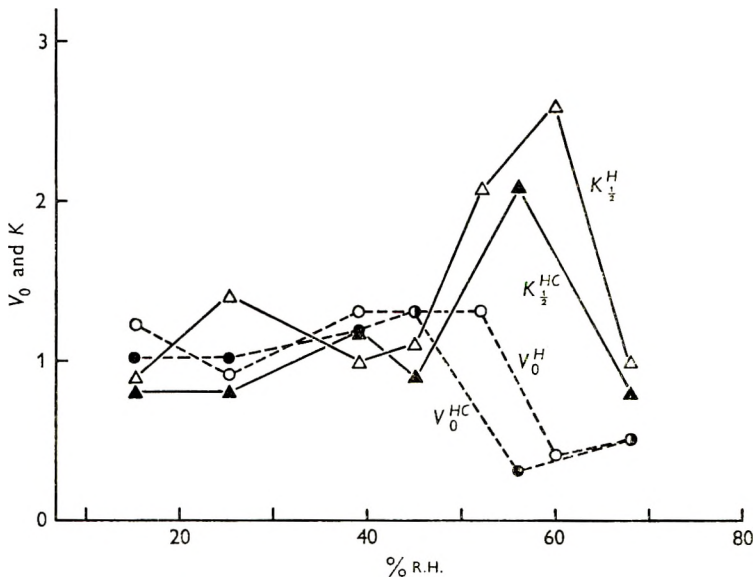

Fig. 6. Decay in air of poliovirus grown in Hanks's balanced salt solution before (*H*) and after (*HC*) incubation with 50–500 μg. L-cystine/ml. for 15 hr. at 37° C. and pH 8. Symbols as in Fig. 3.

After dilution 1/2 in PBS both virus preparations were subsequently tested for their stability in air (see Fig. 6).

Treatment with L-cystine apparently does not change the rate of inactivation of poliovirus in air with varied R.H. This observation is in accordance with our hypothesis that protein is not primarily involved.

DISCUSSION

The kinetics

The relation between virus inactivation and R.H., as depicted in Figs. 3 and 6, is characterized by (1) a maximal spray-loss at low R.H., (2) stability of the

virus after spraying in dry and in wet air, but quick inactivation at moderate humidity, and (3) coincident increase of the K-value with the decline of V_0.

These results confirm the reports of Hemmes *et al.* (1960, 1962) and Harper (1961, 1963). They are also consistent with a tentative rule, deduced from published data on the survival of viruses in aerosols. This rule states that, after spraying in protein-containing suspensions, lipid-free viruses decay with a higher rate at relatively low R.H.'s, whereas lipoviruses die off quickest in relatively wet air (de Jong, 1967); this rule was already suggested by Buckland & Tyrrell (1962) on the basis of experiments with virus suspensions dried on glass slides.

Further, facts 1 and 2 suggest that the virus is inactivated during the desiccation process but is stable when equilibrium is reached, whatever the degree of water loss. At first sight this seems at variance with the phenomena at moderate R.H. However, in these aerosols the inactivation rate decreases with time and eventually levels off to low values, suggesting that the virus is again stable at equilibrium. Although the rate is slowed down, inactivation continues longer, extending beyond the 5 min. of the first sample. This of course implies a decrease of spray-loss with a corresponding increase of storage-loss. These results would consequently fit into the hypothesis of inactivation during dehydration, provided that evaporation of the aerosol droplets and virus particles to equilibrium conditions could be such a slow process. In this context equilibration means equilibrium between the water vapour in the air, the (bound) water in the droplet or droplet nucleus, and the bound water of the virus particle itself. In the authors' knowledge there are no data available about this process in aerosols.

The presence of serum in the spray fluid will slow down evaporation owing to protein hydration. Maximal K-values will then occur at lower R.H. than in the absence of serum. This is indeed what happens as can be seen by comparing Figs. 3 and 6.

The mechanism

Our experiments provide evidence that the virus nucleic acid is primarily affected. Which process is induced or enhanced by desiccation is not known.

Oxidation is an important factor in the thermal inactivation of poliovirus in aqueous suspension at 50° C. (Pohjanpelto, 1958) but not at 37° C. or lower (Lund & Lycke, 1961; Pohjanpelto, 1962). The experiments (at 20° C.) shown in Fig. 4 exclude oxygen as a harmful agent in air.

Though chemical reactions with medium components cannot be ruled out, denaturation seems the most probable mechanism. This is consistent with thermodynamic data (see Table 2).

The low changes of enthalpy and entropy are characteristic for a single chemical event such as the denaturation of single-stranded RNA and exclude protein denaturation as the first event of inactivation. For poliovirus no data are available, however.

If inactivation of poliovirus in aerosols is a denaturation of the RNA, analogy can be suspected to the thermal decay in aqueous solution, as recently analyzed by Dimmock (1967). Dimmock clearly demonstrated that above 44° C. the antigen

structure of poliovirus changes from type 'N' to type 'H' whereas the amount of extractable infectious RNA is unaffected. On the other hand, below 44° C. only the titre of iRNA decreases on prolonged heating, whereas the N-antigen remains intact. In accordance with these observations L-cystine protects poliovirus completely against inactivation at 50° C., very poorly at 37° C. and not at all at 32° C. (Pohjanpelto, 1962).

Table 2. *Activation-enthalpy and -entropy at several modes of virus inactivation*

	ΔH*	ΔS†	ref.‡
Inactivation lipid free viruses in air	8·3	−42	1
Denaturation single stranded virus RNA	19 to 23	−11 to −19	2
Denaturation protein	33 to 200	19 to 537	3
'Low-temperature' heat inactivation poliovirus	28	7	4
'High-temperature' heat inactivation poliovirus	244	689	4

* ΔH = change in enthalpy in kcal./mol.
† ΔS = change in entropy in cal./mol. °K.
‡ References: (1) own calculations; (2) Ginoza *et al.* (1964) with TMV and phage R 17; (3) Woese (1960); (4) Dimmock (1967).

Our findings agree with the phenomena of heat inactivation at low temperature. We suggest that the decay of poliovirus in aerosols proceeds by the mechanism of thermal inactivation of the 'low temperature type', accelerated by dehydration. The accelerating effect of desiccation is intelligible in terms of the concept of stabilizing water molecules. When such a stabilizing water molecule is removed the chance of its replacement by another water molecule is less likely in a dry environment.

SUMMARY

Poliovirus type 1, strain LSc2ab, was directly sprayed in a static air cabinet at 20° C. and sampled afterwards with impingers. Virus inactivation during spraying was maximal in dry air; during storage maximal decay occurred at moderate humidity.

With regard to the mechanism of the inactivation the following facts have been observed: (1) The RNA in the virus decays simultaneously with the whole virus particle. (2) Oxidation does not play any significant role. (3) Incubation with L-cystine protects the virus against inactivation at 50° C. in aqueous suspension, but not against decay in aerosols.

On the strength of these observations it is concluded that in aerosols denaturation of the viral RNA is the cause of the inactivation of poliovirus. The decay in aerosols has some features in common with the thermal inactivation in aqueous suspensions at room temperature.

The technical assistance of Mr M. Harmsen is gratefully acknowledged.

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Bacteriological aspects of air-conditioning plants

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It has been shown in hospitals that positive ventilation in operating theatres may cut the risk of sepsis (Blowers, Mason, Wallace & Walton, 1955; Shooter, Taylor, Ellis & Ross, 1956) and is of value in the treatment of burns (Lowbury, 1954). Many other zones in the hospital could benefit from the supply of clean air, e.g. ward areas, intensive care units and laboratories, and although it has yet to be proved that this would cut down the risk of cross-infection, it would seem beneficial. Ventilation may also be necessary owing to architectural requirements as in deep plan buildings and also to provide comfortable conditions. Ventilation systems are being provided more frequently in hospitals and there is a need for data on the hazards and usefulness of air-conditioning systems in providing clean air.

The Hairmyres Experimental Ward (Report, 1964) has given an excellent opportunity for the study of air-conditioning systems. The 'race track' ward is supplied by four ventilation plants, each different in design. The air-conditioning plant is supplied with fresh air or with a mixture of fresh air and air recirculated from the inside environment. The air-conditioning system is composed of a primary filter, preheater, fan, humidifier and cooling coils, secondary filter, heater battery and supply ducting.

Filtration efficiency. The Medical Research Council Sub-Committee for Operating Theatre Hygiene (Report, 1962) has suggested that filtration down to 5 μ should be adequate for filtration of the air supplied to operating theatres. This recommendation was based on work by Blowers & Crew (1960), who showed that this standard was quite adequate for a full fresh-air system. However, this conclusion has been questioned and no data exist on the efficiency of filtration appropriate to other parts of the hospital. The question of the efficiency of filtration for recirculating air has not been investigated. There is also the possibility that some filters could support the growth of bacteria.

Humidification. Attention has been brought to the bacteriological hazards in air-conditioning humidifiers which use recirculatory tanks (Blowers, Lidwell & Williams, 1962; Shaffer, 1963). Organisms from the pseudomonas, proteus and staphylococcus genera have been incriminated; the primary source of these organisms being the air passing through the sytsem. They may then multiply in the water and are carried into the atmosphere of the room being air conditioned.

Duct surfaces. It has been suggested that dust and bacteria building up inside the air-conditioning ducts may be a potential hazard.

In this study the potential hazards and uses of filters, humidifiers and ducts have been assessed.

MATERIALS AND METHODS

Air-conditioning equipment

At the Hairmyres Experimental Ward four ventilation plants were installed to serve the four zones of the ward. Since two of these plants are similar in construction only three plants were studied. These were those serving the Central Core, Ward and Intensive Care Areas. Built to supply different areas of the ward unit and to enable assessment of various designs, they were provided with different

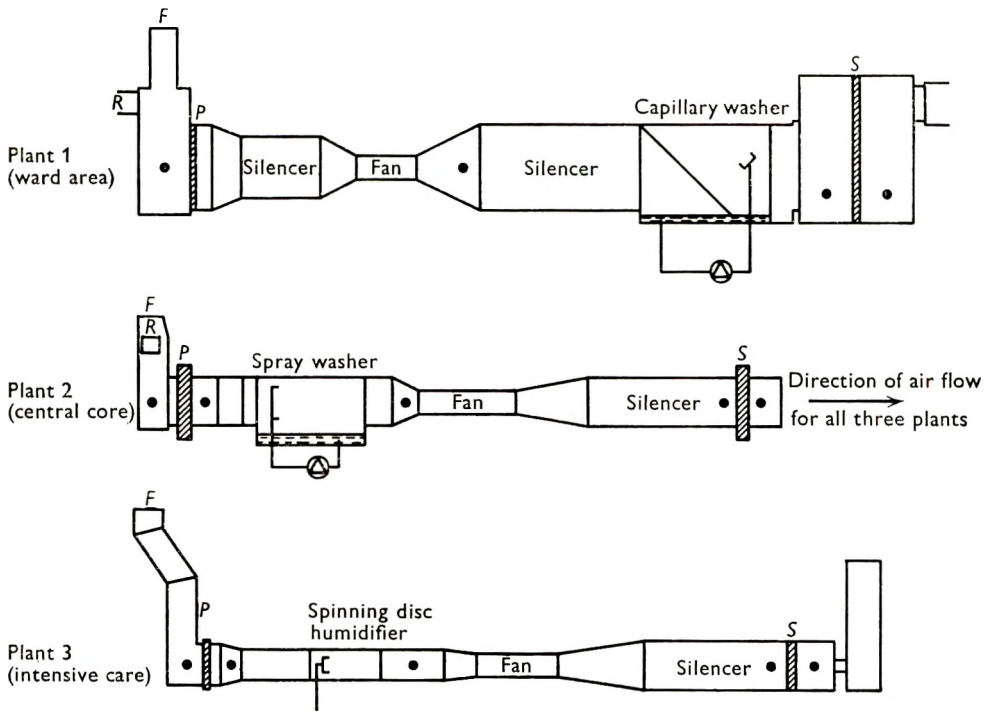

Fig. 1. Diagram of the three air-conditioning plants. ●, sampling point; *P*, primary filter; *S*, secondary filter; *F*, fresh-air duct; *R*, recirculatory duct.

equipment. The three plants are set out schematically in Fig. 1, and will be referred to as plants 1, 2 and 3. As can be seen, each plant has a different type of humidifier. These, as supplied by Copperad Ltd., were a capillary washer, a spray washer and a spinning disk humidifier.

The types of filters in each of the three plants studied are shown in Table 1. The dust-retaining efficiency under specific test specifications of these filters is given in Table 2. Also included are efficiency figures for the Vokes K 600 Kompak, the filter studied by Blowers & Crew (1960). The size distribution of the three dusts conforming to B.S. 2831 are as follows. Test Dust no. 1 includes particles

within the range 0·2 –2 μ in diameter, about 50 % by weight of these particles being over 0·5 μ. Sixty to seventy per cent by weight of the particles of Test Dust no. 2 are within the range 3·5–7 μ in size, 99·5 % of them finer than 13 μ and 2 % finer than 2·5 μ. In Test Dust no. 3, 60–80 % by weight of the particles are within the range 15–25 μ in size, 99·5 % finer than 35 μ and 2 % finer than 10 μ.

The air intakes were placed 23 ft. above ground level and situated towards the centre of the top of the building. The exhausts were 52 ft. from the intakes at each end of the top of the building.

Table 1. *Types of filter in the three air-conditioning plants studied*

Air-conditioning plant	Primary filter	Secondary filter
Plant 1 (ward area)	Vokes Supervee	Under full fresh air– Vokes Univee Grade C Under recirculation– Vokes Univee Grade A
Plant 2 (central core)	Vokes Miniroll Standard	Vokes Miniroll Standard
Plant 3 (intensive care)	Vokes Supervee	Vokes Univee Grade C

Table 2. *Dust retaining efficiency of filters (expressed as the percentage of dust retained by the filters)*

(Data derived from Mulcaster & Stokes 1966)

	B.S. 2831 specification		
Type of filter	Test Dust no. 1	Test Dust no. 2	Test Dust no. 3
Vokes Supervee	5–10	93·5	50·6
Vokes Univee Grade A	75–82	99·35	99·0
Vokes Univee Grade C	18–37	91·8	26·0
Vokes Miniroll Standard	10–30	87·5	68·0
Vokes K 600 Kompak	20–40	97·4	46·6

Bacteriological analysis of air

Bacteriological analysis of air was by means of Andersen samplers (Andersen, 1958). These were placed at the positions shown in Fig. 1, on their sides and facing the air stream. The entry cone usually supplied with this sampler was dispensed with, as this cone causes heavy loss of larger particles on the top sieve (May, 1964). A hinged lid was substituted. The samplers were loaded in an air-conditioned room, only the person filling the sampler being present. This person was capped, masked, gowned and wore sterile surgeon's gloves. The samplers were then placed in the ducts in their appropriate positions with the lids shut. The air-conditioning system was allowed to run for 10 min. in order to clear out any residual contamination. The lid was then pulled back and a 15 cu.ft. sample taken from all six samplers simultaneously. Isokinetic sampling was not considered necessary. Counts were made of the total number of micro-organisms, the total numbers of aerobic bacteria (henceforth known as total bacterial count), *Staphylococcus aureus* and *Clostridium welchii*. Phenolphthalein diphosphate medium (Barber & Kuper, 1951) was used for total micro-organism counts, total bacterial counts and for presumptive counts of *Staph. aureus*. The presumptive *Staph. aureus* were coagulase tested and phage typed.

The size distribution of the micro-organisms was calculated graphically. The percentage cumulated counts by Andersen stages were calculated on a 'less than stated size' for each sampler stage and were plotted on probability scale × 3 cycle log paper or probability scale × 2 cycle log paper, setting the plot point, in microns, as the $D_{50\%}$ of the next stage above. The values for 50 % cumulative particle impaction ($D_{50\%}$) per stage were obtained from published results (Kethley, Cown & Fincher, 1962) and given in Table 3 in terms of equivalent particle diameter, where the equivalent particle is the diameter of a sphere of unit density which has a settling rate in air equal to that of the particle in question.

Table 3. *Fifty per cent cumulative particle impaction per stage of an Andersen bacterial sampler*

Andersen stage	Size of 50% cumulative impaction (μ)
1	9·8
2	6·2
3	3·8
4	2·2
5	0·9

From the line of best fit drawn through the points the equivalent median diameter may be obtained by reading the particle size opposite 50%. The geometrical standard deviation (σg), which is the antilog of the slope of the distribution plot, may be derived directly from the graph by the relationship

$$\sigma g = \frac{84{\cdot}13\,\%\text{ size}}{50\,\%}(\mu) = \frac{50\,\%\text{ size}}{15{\cdot}87\,\%\text{ size}}(\mu).$$

A modification of the medium described by Lowbury & Lilly (1955) was used for the isolation of *Cl. welchii*. This consisted of two layers, a bottom layer of 12 ml. of agar base and a top layer of 15 ml. of supplemented agar. These proportions were finally arrived at as it was found that in order to have the even layer necessary for the Andersen sampler a large top layer was required. The agar base consisted of 2 % Evans peptone with 1·5 % 'Ion agar'. 1·5 % 'Ion agar' was finally decided upon, as 4 % agar was too thick to dispense at 55–60° C. when egg-yolk emulsion was present. It was also found that proteus and other swarming organisms did not occur often enough to be a problem. The supplemented agar was prepared by adding to 100 ml. of agar base 1·5 ml. neomycin (10,000 μg./ml.) (Upjohn), 6·5 ml. Fildes extract (Oxoid), and 10 % egg-yolk emulsion (Oxoid). Ten per cent egg-yolk emulsion was decided upon as this produced a better Nagler reaction than either 5 % or 1 %, although these were fairly satisfactory.

This medium was made both with and without neomycin. This was because it was known (Dr E. S. Broughton, personal communication) that a heat-treated suspension of *Cl. welchii* (i.e. only spores) did not grow well on neomycin medium. However, it appeared that neomycin did not significantly influence the counts of the small number of *Cl. welchii* obtained. The results obtained from plates with neomycin were pooled with the counts from plates not containing neomycin.

Bacteriological analysis of surfaces

The method used was that of swabbing with alginate swabs (Higgins, 1950). Using a masking plate, a 2 in. diameter circle was swabbed three times. This method was used in preference to others, e.g. impression plate, because of a layer of dust at many of the sampling points. The three swabs were dissolved in 30 ml. of fluid and 0·5 ml. samples plated out on each of two aerobic plates and two anaerobic plates (one with neomycin, the other without).

Contamination of the humidifier and air sampling

Bacillus subtilis var. *globigii* was used. This organism produced a yellow-orange colony which could be easily identified. The bacteria were cultured in 16 oz. medical flat bottles containing 100 ml. of 2·5 % peptone water and 0·5 ml. of Tween 80. About 40 bottles were used and these were laid on their sides. The Tween 80 was added to prevent pellicle formation. The bacteria were incubated at 37° C. for 7 days, centrifuged and resuspended in water. The centrifugation was necessary because humidifiers foamed with the broth and Tween, and the foam set free gave erroneous results. The bacteria were added to the humidifier in various dilutions and the water sampled for *B. subtilis* var. *globigii*. Three samples were taken and two sets of plates used for each. The air was sampled using the same method as for the filters but with Andersen samplers after the humidifiers and the secondary filters.

All bacterial samples throughout the experiments were incubated at 37° C. and counted after 36 hr.

RESULTS

Humidifiers

The number of bacteria given off by recirculatory type humidifiers

The concentration of the test bacteria, *B. subtilis* var. *globigii*, in the air after the humidifier, as compared to the concentration in the humidifier reservoir, is given in Table 4. The air sample was limited to 15 cu.ft. to avoid overdrying of the agar. Because of this it was not practicable to take tests with lower concen-

Table 4. *Concentration of* Bacillus subtilis *var.* globigii *emitted from two humidifiers compared with the amount in the reservoir*

(One test is a 15 cu. ft. sample of air,)

Humidifier type	No. of tests	Bacterial count/ml. water in reservoir	No. of bacteria per cu.ft. of air
Spray type	6	$3{\cdot}0 \times 10^3$	0·189
	4	$5{\cdot}2 \times 10^3$	0·213
	10	$9{\cdot}0 \times 10^3$	0·62
	6	$8{\cdot}6 \times 10^4$	1·335
	12	$2{\cdot}4 \times 10^5$	3·32
Capillary washer	6	$2{\cdot}5 \times 10^3$	0·100
	6	$3{\cdot}0 \times 10^3$	0·133
	8	$5{\cdot}8 \times 10^4$	0·732
	6	$3{\cdot}5 \times 10^4$	0·80

trations than those shown. These results were plotted on a graph and because of the similarity of results between the two humidifiers it was decided to combine the results as one. This similarity in results is probably because of the similarity of the design features which would be expected to influence the number of bacteria emitted, i.e. elimination plates, spray nozzles and water supply rate.

The results as in Table 4 were analysed to establish the relation between the two variables. By means of regression analysis the relationship was derived in two ways. First, the line of best fit was established. This is:

$$y = 0{\cdot}0000129x + 0{\cdot}213,$$

where y is the concentration of bacteria emitted from the humidifier per cu.ft. of air after the humidifier and x is the concentration of bacteria per ml. of water in the reservoir of the humidifier. The correlation coefficient is 0·99. Since it must be assumed that the line passes through the origin, i.e. when there are no bacteria in the humidifier reservoir no bacteria are given off, the data were recalculated. The equation for the line passing through the origin is

$$y = 0{\cdot}000014x$$

and the correlation coefficient is 0·989.

This line, which has a high correlation coefficient, is regarded as the relation between bacterial concentration in the reservoir of the humidifier and the number of bacteria given off into the air.

Size of particles emitted from the humidifiers

From the number of *B. subtilis* var. *globigii* obtained on the Andersen sampler stages it was calculated in the case of the spray washer that the equivalent median diameter was 2·2 μ. This was calculated from data of 16 tests, i.e. 240 cu.ft. of air sampled. Similarly, it was found from 14 tests that the equivalent median diameter of the bacteria being emitted from the capillary washer was 1·7 μ.

The effect of filtration by Vokes Univee Grade 'C' filters on bacteria given off by the humidifiers

On sampling a total of 210 cu.ft. of air before and after the secondary filter in the plant it was found that of the 162 *B. subtilis* var. *globigii* given off by the humidifier 71 bacteria passed through the filter. This is a removal efficiency of 56%.

It was also found that on testing the air in the area supplied by the plant, 4 hr. after the bacteria had been added to the humidifier water, a count of 0·5 bacteria/cu.ft. was obtained with four 15 min. tests. This compared to a figure of 2·8 bacteria/cu.ft. leaving the humidifier. The bacterial content of the water at that time was $2{\cdot}4 \times 10^5$/ml.

Concentration of bacteria in the humidifier reservoirs under normal conditions

Samples of the humidifier reservoir water were taken periodically over a year. With one exception, the concentration of bacteria in the samples taken never exceeded 40 bacteria/ml. The mean concentration was 21 bacteria/ml. The two

humidifiers usually had a constant overflow of water to dilute the bacteria and dust washed out of the air, but on one occasion the overflow stopped working. This was for a period of at least 4 weeks and on this occasion the bacterial concentration rose to $2{\cdot}05 \times 10^3$ bacteria/ml. before steps were taken to remedy the fault. These bacteria which were in almost pure culture were shown to be small Gram-positive rods probably *Corynebacterium* species and not thought to be potential pathogens.

Bacterial contamination of duct surfaces

Only Plant 1 was studied which had as a secondary filter a Univee Grade 'C' for full fresh air and a Univee Grade 'A' for recirculation. The results are considered in three groups. These consist of:

(*a*) The number of bacteria on the ducting from, and including, the extract grilles in the ward area to either the outside environment or the point of mixing with the fresh air before the primary filter. These are known as the recirculatory ducts.

(*b*) The number of bacteria on the supply ducts when full fresh air was being supplied.

(*c*) The number of bacteria on the supply ducts when two-thirds of the air was being recirculated.

The results are presented as total bacterial counts, *Cl. welchii* counts and *Staph. aureus* counts or, in the case of the recirculatory ducts, presumptive *Staph. aureus* counts.

Recirculatory ducts

Thirteen sampling points were chosen, four were at extract grilles, the remaining nine being positioned throughout the recirculatory duct system up to the point in front of the primary filter where the mixing of the fresh and recirculatory air took place. Approximately ten samples were taken at each position, 139 samples in all. The mean total count of bacteria was $1{\cdot}7 \times 10^3$/sq.in. of duct surface. This varied from $3{\cdot}4 \times 10^3$ bacteria/sq.in., the count at the extract grilles, to $1{\cdot}54 \times 10^2$ bacteria/sq.in. at the point before the secondary filter. This tendency to higher counts at the beginning of the recirculatory system falling by a 10 times reduction at the end of the system was reflected throughout the system—the further one went through the duct system the lower the count became. This was probably caused by two factors. It would be expected, because of impaction, that the concentration of particles would decrease down the duct system. It would also be expected in the larger ducts, apart from the lower concentration in the air, that the lower velocity should lead to less impaction. Anaerobic counts of *Cl. welchii* gave a mean count of 25·3 bacteria/sq.in., the range being from 0 to 193 bacteria/sq.in. Presumptive *Staph. aureus* counts accounted for approximately $\frac{1}{100}$ of the total bacterial counts, irrespective of the position at which the sample was taken.

Supply ducts with 100 % *fresh air*

Samples were taken before the primary filter and at various places throughout the supply system. A mean total count of $3{\cdot}66 \times 10^2$ bacteria/sq.in. was obtained before the primary filter. No *Staph. aureus* were isolated and the mean count of *Cl. welchii* was 9·5/sq.in.

The average count throughout the supply side, i.e. after the secondary filter, was 20 bacteria/sq.in. No *Staph. aureus* were isolated and out of 31 tests two *Cl. welchii* were isolated.

Supply ducts with two-thirds recirculation

Samples, taken at the same locations as used for the supply ducts with 100 % fresh air, showed an almost identical pattern. A total bacterial count of $1{\cdot}54 \times 10^2$/sq.in. was obtained before the primary filter, but a few *Staph. aureus* and *Cl. welchii* could be isolated. The *Cl. welchii* count was 20/sq.in. After the secondary filter the total bacterial count was 10/sq.in. Only two *Cl. welchii* and one *Staph.*

Table 5. *Air sampling throughout the air-conditioning plants*

		Air-conditioning plant					
		Plant 1 (ward area)			Plant 2 (central core)		Plant 3 (intensive care)
Amount of fresh air (%)		100	33 (C)*	33 (A)†	100	75	100
No. of tests		60	60	60	40	30	40
Intake	B	0·42	2·16	1·93	0·485	0·873	0·985
	B + A + M	1·71	3·14	2·08	2·51	3·21	4·21
After primary filter	B	0·071 (16·9)	0·557 (25·8)	0·424 (22·0)	0·064 (13·1)	0·146 (16·7)	0·152 (15·4)
	B + A + M	0·563 (32·9)	1·000 (31·8)	0·885 (42·5)	0·845 (33·7)	1·450 (45·2)	2·800 (66·5)
After humidifier	B	N.D.	N.D.	N.D.	0·061 (12·5)	0·091 (10·4)	0·096 (11·1)
	B + A + M	N.D.	N.D.	N.D.	1·050 (41·8)	0·854 (26·6)	1·665 (39·5)
Before secondary filter	B	0·035 (8·3)	0·318 (14·7)	0·183 (9·5)	0·061 (12·5)	0·121 (13·9)	0·105 (10·3)
	B + A + M	0·320 (18·7)	0·700 (22·3)	0·264 (12·7)	0·970 (38·7)	0·994 (31·0)	1·615 (38·4)
After secondary filter	B	0·035 (8·2)	0·198 (9·2)	0·013 (0·7)	0·046 (9·5)	0·079 (9·0)	0·091 (9·2)
	B + A + M	0·237 (13·9)	0·494 (15·7)	0·021 (1·0)	0·790 (31·5)	0·972 (30·3)	1·290 (30·6)

Mean bacteria counts (B) and mean micro-organism counts (B + A + M) are expressed as numbers per cu.ft. Figures in parentheses indicate percentage penetration. One test was 15 min. or 15 cu.ft. of air. N.D. indicates not done.

* Final filtration through grade C filter.

† Final filtration through grade A filter.

aureus were obtained out of 31 tests. More attention was paid to the supply diffusers and grilles, as evidence had been obtained suggesting a build-up of micro-organisms there. A mean bacterial count of 127/sq.in. and a mean *Cl. welchii* count of 10/sq.in. was obtained from 12 samples.

Penetration of the air-conditioning plants by bacteria and other micro-organisms

Total counts

The results obtained by air sampling at the points of the air-conditioning plants shown in Fig. 1 are given in Table 5. The counts per cu.ft. of bacteria (total bacterial count) and bacteria together with actinomycetes and moulds (total micro-organism count) are given. These counts are expressed also as the percentage penetration of the organisms with respect to the initial concentration.

The air samples were made up at times with large numbers of actinomycetes, which were morphologically distinct and could be easily picked out by their high phosphatase activity and characteristic colonial appearance. These actinomycetes were found mainly on stage 5 of the Andersen sampler and had an equivalent median diameter of $1{\cdot}5\,\mu$. These organisms arrived at various concentrations and at various times; concentrations as high as 14/cu.ft. were obtained when the total bacterial count was 1·8/cu.ft. Moulds, although not so erratic in their appearance in the air, had also a small equivalent median diameter ($3{\cdot}5\,\mu$). The total micro-organism counts shown in Table 5 have, therefore, an erratic nature, and owing to the nature of the size distribution the air-conditioning systems were much less effective in removing them. Approximately 30 % of the total micro-organism count could pass through the air-conditioning system compared to approximately 9 % of bacteria.

The sampling point after the humidifier and before the secondary filter should give similar results, as the only obstruction between these points is a silencer. The results show reasonable similarity both with total micro-organism counts and total bacterial count which reflects well on the experimental reliability. In the case of plant 1, sampling after the humidifier was not possible owing to the inaccessibility of this part of the plant.

Staphylococcus aureus *counts*

These bacteria were not found to any large extent in the air. The mean count in the fresh air was 0·008/cu.ft. and in the recirculated air before the primary filter was 0·04/cu.ft. Occasionally throughout the three plants *Staph. aureus* could be isolated under all ventilation schemes, but few *Staph. aureus* were isolated after the secondary filter in any of the plants. In plants 1 and 3, under full fresh air, no *Staph. aureus* were isolated after the secondary filter but in plant 2 two *Staph. aureus* were isolated. Under recirculation in plant 3 two *Staph. aureus* were isolated whereas in plant 1 filtration through a Grade 'C' filter gave three *Staph. aureus* and filtration through Grade 'A' one. These *Staph. aureus* were the total number found throughout the tests. These are in line with the level which could be expected considering the initial concentration before the plants and the filtration efficiency of the plant.

Clostridium welchii *counts*

Tests were only carried out in plant 1 before the air-conditioning plant and after the secondary filter under two-thirds recirculation and full fresh-air ventilation. Fifty tests of 15 min. were carried out in each case. The concentration before the plant was 0·017/cu.ft. both with full fresh air and with two-thirds recirculated air. No *Cl. welchii* were found after the secondary filter.

Contamination of a secondary filter

In plant 1 it was found that the secondary filters had been overgrown with mould. The filter bags were wet to touch and it was apparent that free water was passing from the humidifier, which was just in front of the secondary filter, to the filter bags. Three 4 in. samples of the material (Vokes Univee Grade 'C') were homogenized and serial dilutions taken of the homogenate on to two plates each of Sabouraud dextrose agar (Oxoid), Oxoid blood agar base no. 2, and *Cl. welchii* medium. The mean counts obtained per sq.in. of material were:

Moulds	$2{\cdot}1 \times 10^5$	Aerobic bacteria	$1{\cdot}1 \times 10^2$
Yeasts	$1{\cdot}0 \times 10^6$	*Cl. welchii*	$1{\cdot}5 \times 10^2$

Although no identification was made of the aerobic bacteria isolated it could be seen from colonial appearance that a large percentage of them belonged to *Bacillus* spp.

Comparison of the size distribution of micro-organisms prior to the air-conditioning plant under full fresh air and two-thirds recirculation

This comparison was made to see if the air-conditioning plants would be subject to a different distribution of micro-organisms from the outside or the recirculated air, which would reflect on the filtration required.

The counts obtained from the individual stages of the Andersen samplers sited at the intake of plants 1, 2 and 3 under full fresh air were analysed to give the size distributions of the micro-organisms in the fresh air. The size distributions of micro-organisms in recirculated air were obtained for plant 1 only. Plant 2 was not tested, since only a quarter of the inside air was recirculated.

Statistical analysis

In order to assess the possibility that the size distributions were different for fresh air and recirculation the χ^2 test was applied. This was done by means of contingency tables and carried out in the case of bacteria, actinomycetes, moulds and *Staph. aureus*. The results are shown in Table 6. From these results, and the use of χ^2 tables, it can be seen that an obvious difference exists in the size distribution of bacteria, a small difference with the actinomycetes and moulds and little or no difference in the *Staph. aureus* size distributions. It must be realized in the case of *Staph. aureus* that the sample, especially with full fresh air, was very small.

Graphical analysis

By cumulative addition and plotting against 50% cut-off values of each Andersen sampler stage the results shown in Table 7 were obtained, as a comparison between full fresh air ventilation in plants, 1, 2 and 3 and recirculation in plant 1. Table 7 gives the equivalent median diameter and the geometric standard deviation of the size distributions of bacteria, *Staph. aureus*, moulds, actinomycetes and the total micro-organisms count, under full fresh air and recirculation.

Table 6. *χ^2 values for the difference in size distribution of micro-organism particles under full fresh air and recirculation*

Bacteria	197·60
Actinomycetes	17·37
Moulds	13·51
Staph. aureus	1·91

Table 7. *Equivalent median diameter (EMD) and geometric standard deviation (GSD), in microns, of micro-organisms in fresh air and recirculated air*

	Bacteria		*Staph. aureus*		Moulds		Actino-mycetes		Total micro-organism count	
	EMD	GSD	EMD	GSD	EMD	GSD	EMD	GSD	EMD	GSD
Full fresh air	16·0	5·3	9·2	4·9	3·6	2·0	1·7	2·3	2·8	1·9
Recirculation	6·4	3·3	8·9	4·2	3·5	1·9	1·6	2·0	4·5	2·6

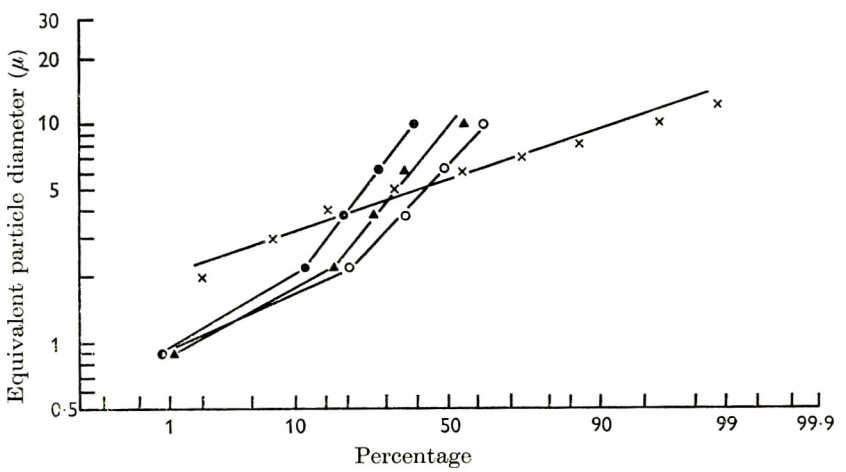

Fig. 2. Particle size distribution of *Staph. aureus* and bacteria under two-thirds recirculation and full fresh air. Also given is the size distribution by weight of Aloxite 50. ●, Bacteria full fresh air; ○, bacteria two-thirds recirculation; ▲, *Staph. aureus* under both fresh air and recirculation; × ——— ×, Aloxite 50.

A log-normal distribution was assumed and it was found that the results conformed very well to this distribution. It was only in the final stage of the sample that the results became truncated and, in the case of the moulds, stage 5. This one would expect at the tail of a log-normal distribution.

In Fig. 2 the size distribution of bacterial particles in fresh air and air of which two-thirds was recirculated are shown along with the size distribution of *Staph. aureus* particles in which size distribution in fresh air and recirculated air have been combined because of their similarity. Also shown is the size distribution by weight of Aloxite 50, a test dust conforming to B.S. 2831, Test Dust no. 2. In Fig. 3 the size distributions of moulds and actinomycetes are given.

It can be seen from this graphical method that under recirculation the medians of the sizes of all the micro-organisms are smaller except in the case of the total count. The differences are fairly small for moulds, actinomycetes and *Staph. aureus*, but the bacteria show quite a considerable difference in size. In the case of total micro-organisms, however, the median size in full fresh air is smaller than that of recirculation. This is because of the relatively larger numbers of larger bacteria present in the recirculated air, compared with the large numbers of smaller actinomycetes and moulds which are present in fresh air. This also shows the confusion that could arise if the actinomycetes had not been recognized and therefore treated separately.

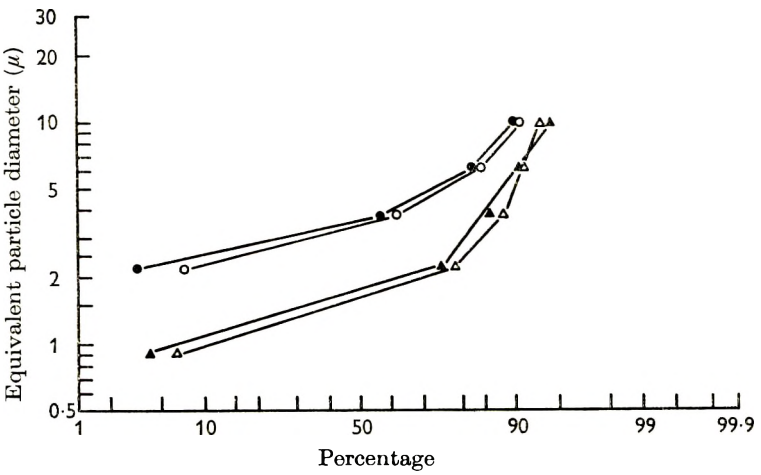

Fig. 3. Particle size distribution of actinomycetes and moulds under two-thirds recirculation and full fresh air. ▲, Actinomycetes full fresh air; △, actinomycetes two-thirds recirculation; ●, moulds full fresh air; ○, moulds two-thirds recirculation.

DISCUSSION AND CONCLUSIONS

Humidification

It has been shown that by the artificial contamination of recirculatory storage tanks and humidifiers, bacterial concentrations can be obtained as given by the equation

$$y = 0{\cdot}000014x,$$

where y is the concentration of the bacteria particles given off by the humidifier per cu.ft. of air passing through the humidifier, and x the number of bacteria per ml. in the reservoir. This equation is only strictly applicable to the humidifiers tested, but it is unlikely that other humidifiers of these types would give results which differed markedly. Although these counts appear low it would not require

a high concentration of bacteria in the reservoir to give a concentration of bacteria in the air which would be comparable to the concentrations which were normally obtained in the air-conditioning system near the humidifier, i.e. counts of approximately 0·1 bacteria/cu.ft. The significance of the number of bacteria emitted from the humidifier is reinforced by the observation of the size and penetration power of the bacterial particles given off. The bacterial particles emitted from the humidifier are of droplet nuclei size, around 2 μ, and between 40 and 50 % of them are able to penetrate filters of filtering efficiency 91·8 % against B.S. 2831 Test Dust no. 2.

In normal circumstances the number of bacteria in the water used by the humidifier was less than 40 per ml., suggesting that the bacteria were not multiplying in the reservoir. This bacterial count was low enough to cause negligible trouble but this was in a well-maintained plant with a good overflow of fresh water through the recirculatory tank. In one case when the overflow was not functioning for a few weeks a count of $2{\cdot}05 \times 10^3$ bacteria/ml. was obtained. Although the bacteria found in this build up were not considered potential pathogens, for hospital purposes a safer method should be used, i.e. one which does not use recirculating water.

Duct surfaces

It has been shown that fairly large numbers of bacteria (an average of $1{\cdot}7 \times 10^3$ bacteria/sq.in. of duct surfaces), with a significant number of pathogens, are present on the extract side of the ventilation system, the numbers decreasing as one goes through the recirculatory system. It has been suggested that filters at each duct extract point should be provided or that regular cleaning should be given to the ducts to prevent these bacteria entering the room. No evidence was obtained to show that this was a significant problem but the design of the ventilation plant to avoid reverse flow should be considered in more critical areas where the ventilation plant may be shut off, e.g. operating theatres and treatment rooms.

The supply ducts of the air-conditioning plant had satisfactorily low counts of bacteria. Under recirculation the bacterial count was 10/sq.in. and under full fresh air 20/sq.in. Only the occasional pathogen was isolated. The exception to this satisfactory state of affairs was the inlet supply grilles and diffusers which showed a higher count of bacteria than the ducts supplying them. This was probably caused by impaction of entrained air from the room being supplied. The concentration of *Cl. welchii* on the inlet grilles and diffusers was significant enough to merit regular cleaning of the inlet points, especially in operating theatres and treatment rooms. The use of terminal filters does not appear to be justified as a bacteriological measure except where they are used instead of diffusers, as in laminar flow systems.

Filtration

The aim of these experiments was to produce data which could be used as design standards for the production of bacteriologically acceptable air in future hospital buildings. The bacteriological quality of air supplied by an air-conditioning plant, as far as filtration is concerned, is dependent on three points: the concentration and size distribution of the micro-organisms in the air to be filtered, and the efficiency of the filters against particles of these sizes.

The three types of microbiological airborne counts used in the evaluation of filters were the total number of aerobic bacteria (total count), the *Staph. aureus* count and the *Cl. welchii* count. Actinomycetes and moulds were excluded from a critical evaluation of the data because of their non-pathogenic nature, their unusually low equivalent median diameter (1·5 and 3·5 μ respectively) and their intermittent presence. Failure to recognize these actinomycetes could have given rise to results which would have underestimated the potential usefulness of filters. It is also possible that the higher counts of micro-organisms in the outside air obtained in some studies may be due to actinomycetes. In this study mean concentrations of 0·6 bacteria/cu.ft. and 2·4 micro-organisms/cu.ft. were obtained in the fresh air. The *Cl. welchii* count found in the outside air was 0·017 particles/cu.ft., whereas the *Staph. aureus* count was 0·008/cu.ft. These counts are in the region of those reported by previous authors, e.g. Blowers & Crew (1960), Lowbury & Lilly (1958), and Report (1948). In the case of recirculated air the bacterial concentration was 2·05/cu.ft., all micro-organisms 2·61/cu.ft., *Cl. welchii* 0·017/cu.ft. and *Staph. aureus* 0·04/cu.ft.

From the plotted distribution of the bacteria given in Fig. 2 the median diameter of bacterial particles in the recirculated air is 6·4 μ, those in the fresh air 16 μ. One of the limitations of the Andersen sampler for normal bacterial sampling is shown in Fig. 2 since only the size distribution of the smaller 50 % of the bacterial sample was obtainable for plotting the distributions. Some loss of larger particles would also be expected on the top sieve of the sampler (May, 1964). In the case of moulds and actinomycetes and also bacterial particles sampled after the filters, almost full size distributions were obtained. These particles were found on the lower stages of the sampler. The number of these particles lost would therefore be extremely small.

It can be seen that the size distributions of bacteria under full fresh air and recirculation converge; the difference between the two diminishing as the size of the bacteria decreases. One would therefore expect that primary filtration, by removing the larger bacterial particles, would tend to reduce the differences between the size distributions of the bacteria. In Table 5 this is substantiated in that 16 % of the bacterial particles in the fresh air penetrate the primary filters in plants 1 and 3, compared with approximately 24 % of the bacterial particles in the recirculated air. This difference is not in itself very great but it can be seen that under both recirculation and full fresh air the penetration of the secondary filters by the bacterial particles is approximately 9 %. In the case of filtration in plant 2 where the recirculated volume is only one-quarter of the total volume, the

figures for penetration through the primary filter are fairly similar, 13·1 and 16·7 %. At the secondary filters the similarity is even more striking with 9·5 and 9·05 % penetration. This evidence suggests that recirculated air and fresh air have size distributions which are similar enough for primary filtration to reduce the difference to practical insignificance. However, owing to the presence of greater numbers of *Staph. aureus* higher standards of filtration are probably advisable.

The three air-conditioning plants supplied air with concentrations of total bacteria and pathogenic bacteria which appeared quite satisfactory. It was found that passing fresh air through an air-conditioning plant which had a final filtration efficiency of 91·8 % to B.S. 2831 Test Dust no. 2 gave a count of 0·035–0·09 bacteria/cu.ft. On recirculation of two-thirds of the ward air and a secondary filter of efficiency of 99·35 % to B.S. 2831 Test Dust no. 2 a mean concentration of 0·013 bacteria/cu.ft. was obtained. Even better removal efficiency than the 99·33 % removal obtained through this filter was probable since in the 60 tests only 12 bacteria were found which had apparently penetrated the filter. The possibility that several of these bacteria were inadvertently introduced into the 360 Petri dishes used seems a real one.

Shown in Fig. 2 along with the size distribution of bacteria and *Staph. aureus* is the size distribution of Aloxite 50, the test dust used in testing filters to B.S. 2831 Test Dust no. 2. The size distribution of this test dust approximates nearest of any of the test dusts to the size distribution of the bacteria and *Staph. aureus*, although it is slightly smaller. It can be seen in Table 5 that the removal efficiency of the primary filters against bacteria is less efficient than against B.S. Test Dust no. 2. If, however, the results of penetration of the secondary filters are considered it can be seen that a fair correlation between the efficiencies is obtained. This is shown in Table 8.

Table 8. *Comparison of the removal efficiencies of the secondary filters against bacteria and Aloxite* 50

Type of filter	Percentage efficiency against bacteria	Percentage efficiency against Aloxite 50
Univee Grade 'A'	99·3	99·35
Univee Grade 'C'	90·8, 91·8, 90·8	91·8
Miniroll Standard	90·5, 91·0	87·5

Similarly, results taken from the work of Blowers & Crew (1960) show that filtration of fresh air through a K 600 Kompak rated 97·4 % efficient to Aloxite 50 was 97·85 % efficient in the removal of bacterial particles. It would seem, therefore, that a fair approximation to the filtering efficiency of a filter to bacteria in the air can be obtained by reference to the filter's quoted efficiency to Aloxite 50; the bacterial removal efficiency being equal to the quoted efficiency. This approximation is dependent on the fairly standard practice of a filter of efficiency approximately 90 % to Aloxite 50 being used as a primary filter.

From the results given it is felt that a filter can be chosen for the purpose in hand. Although standards may vary from situation to situation depending on

several variables, the author would give the following standards for guidance. Primary or pre-final filtration is assumed to be approximately 90% to B.S. 2831 Test Dust no. 2.

	Fresh air %	Recirculated %
Non-critical areas, e.g. general wards, corridors, etc.	90	99
Critical areas, e.g. operating theatres, treatment rooms, isolation rooms, etc.	99	> 99

The percentage figures are bacterial removal efficiency figures or the quoted efficiency of the filter to Aloxite 50.

It was found, in plant 1 only, that moulds and yeast but no bacteria were growing in the secondary filter. This was because of the close proximity of the humidifier to the filter. This could be overcome by either moving the filter away from the humidifier, using more effective means of eliminating the water carried over from the humidifier, or by placing the heater batteries in front of the secondary filter (this would be impractical with multizone reheat).

No experimental observations were made of the ability of viral particles to pass through filters, and no work on this topic appears to have been published. It is probable that viral particles are not of viral size but are released from humans accompanied by body materials; it is also known that some viruses lose their viability quickly (Tyrrell, 1967). It seems possible, therefore, that normal standards of filtration may suffice. Owing to lack of sources and the large amount of dilution which will occur if viruses are dispsersed into the outside air, it is doubtful if fresh air contains many viruses. Until more information is made available it is suggested that in areas where viral infection is considered a problem ventilation should be by fresh air, or the area concerned be regarded as 'critical' for the purposes of filtration of recirculated air.

From the data given it is felt that the air-conditioning plants in the experimental ward did all that was required of them in producing air for use in the ward area and it is hoped that the results obtained can be used in other hospital situations.

SUMMARY

An investigation was carried out into the bacteriological performance of three air-conditioning plants in a hospital ward. Two of these plants had the facility for recirculating part of the ward air.

An equation has been derived comparing the concentration of bacteria which would be expected to be given off by the humidifiers in the ventilation system, with the concentration of bacteria in the recirculatory tank. The bacterial particles given off by these humidifiers were of nuclei droplet size, and were found to penetrate the filters used with a fair degree of ease. Although the number of bacteria in the humidifier water remained insignificant with a constant overflow of water into the recirculatory tank, on one occasion a build-up of bacteria was demonstrated when the overflow ceased. For hospital use humidifiers of a non-recirculatory type should be used.

The concentration of bacteria on the surface of the recirculatory ducts was

assessed, as also were those on the surface of the supply ducts under full fresh air and recirculation. The concentration of bacteria in the supply ducts was low and the use of terminal filters was not merited, although care should be taken to prevent the build-up of bacteria in inlet grills and diffusers. The bacterial concentration in the exhaust ducts was found to be quite high. It was therefore thought that in critical areas, where the ventilation plant may be shut off, the use of some device to prevent reversed air flow may be necessary.

The count of various types of micro-organisms in the fresh air and two-thirds recirculated air are given along with their size distribution. The results of the effect of filtration on the concentration of bacterial particles throughout the air-conditioning plant is given under full fresh air and recirculation. These concentrations appear quite satisfactory. It was found that one set of filters had been overgrown by mould because of free water being brought over from the humidifier. Measures have been suggested to overcome this. When primary or prefinal filtration was approximately 90 % efficient to Aloxite 50 (B.S. 2831 Test Dust no. 2) it was demonstrated that a fair approximation to the final filtration figure could be obtained by reference to the quoted efficiency of the final filter to Aloxite 50. After similar primary filtration it was demonstrated that the final filtration of filters against recirculated and fresh air was approximately the same. Owing to the higher number of *Staph. aureus* in recirculated air, higher efficiency filtration may be required.

Standards of filtration efficiency for critical and non-critical zones are suggested.

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Immunological response in cows to *Mycoplasma bovigenitalium*, strain 1836

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INTRODUCTION

The occurrence of contagious bovine pleuropneumonia (CBPP) in cattle in France (FAO, 1967) made it advisable to become familiar with diagnostic procedures for CBPP. CBPP reagents consisting of boiled *Mycoplasma mycoides* antigen, positive CBPP bovine serum and negative bovine serum were obtained from the Animal Health Research Laboratory, Parkville, Australia. Bovine serum samples were obtained from the Laboratory herds and from herds in various parts of England and Wales. Serum samples, taken from cattle affected by a wide range of pathological conditions, were also examined. The complement-fixation (CF) test used was that described by Campbell & Turner (1963). The results showed that one serum sample out of a total of 210 examined gave a significant reaction: at the 1/10 dilution almost complete fixation occurred, at the 1/20 dilution partial fixation and at 1/40 slight fixation. According to Campbell & Turner (1963), this reaction would have been classified as a weak positive which is usually associated with early cases of CBPP, chronic cases with small old sequestra or recovered cases with pleural adhesions or fibrosis.

The positive serum sample had been taken from a cow experimentally infected 3 weeks previously via the teat canal with *Mycoplasma bovigenitalium* (Stuart *et al.* 1963). A serological reaction between sera from *M. bovigenitalium* infected cows and *M. mycoides* antigen had been observed by Priestly using the agglutination test in Africa (R. H. Leach, personal communication). Roberts & Olesiuk (1967) showed that sera from chickens infected with *M. synoviae* reacted with *M. gallisepticum* antigens, whereas sera from chickens infected with *M. gallisepticum* did not react with *M. synoviae* antigens. Roberts & Olesiuk (1967) also showed that when *M. synoviae* was inoculated into birds, anti-γ-globulin activity was stimulated in the serum, and that *M. gallisepticum* antigens were sensitive to this anti-γ-globulin activity. Dr D. G. ff. Edward (personal communication) suggested that a relationship may exist between *M. bovigenitalium* and *M. mycoides* similar to that existing between *M. synoviae* and *M. gallisepticum*.

The aim of the present study was to investigate the immunological response of cattle to *M. bovigenitalium* and to study the serological relationship between *M. mycoides* and *M. bovigenitalium*.

MATERIALS AND METHODS

Mycoplasma bovigenitalium, strain 1836, was used, isolated from an outbreak of bovine mastitis (Stuart *et al.* 1963).

Media

The broth medium consisted of Difco PPLO broth, enriched with 10% inactivated horse serum, 1% yeast autolysate (Albimi), 0·2% dextrose, 1000 units penicillin G/ml., pH 7·8. Sloppy agar (agar concentration 0·04%) and agar plates were prepared with this medium using Oxoid Ionagar no. 2.

Animal inoculation

Six cows were used, varying from 2·5 to 6 years of age. Four were in full milk production while two were dry. Before inoculation all were tested serologically and culturally for evidence of infection with *M. bovigenitalium* and *M. mycoides*.

The six cows were inoculated with a broth culture of *M. bovigenitalium*, strain 1836. The four cows in milk were inoculated via the teat canal into the left fore and hind quarters, each quarter receiving $8{\cdot}1 \times 10^8$ organisms as judged by plate counts. One dry cow was inoculated via the nasal cavity and the other via the vagina, each cow receiving $8{\cdot}1 \times 10^8$ organisms.

Blood samples and milk samples were taken daily for 3 weeks, and thereafter at regular intervals. On each occasion a heparinized blood sample and a clotted blood sample were taken. Milk samples were taken on each occasion at approximately the same time, about 30 min. after morning milking. Attempts were made to isolate *Mycoplasma* from the heparinized blood and milk. Serum was prepared from the clotted blood and subjected to the serological techniques described below.

Isolation of mycoplasmas and their identification

The milk samples and heparinized blood samples were cultured in a similar manner. Two ml. of the sample was inoculated into sloppy agar and the sample cultured on PPLO agar. The agar plates were incubated in a moist atmosphere at 37° C. The sloppy agar cultures were incubated for 5–6 days, and then subcultured into sloppy agar and on agar plates.

Mycoplasma strains were cloned from single colonies, and were then identified using the growth inhibition test (Clyde, 1964). Broth cultures were plated on PPLO agar using cotton-wool swabs. Sterile 6 mm. antibiotic assay disks (W. and R. Balston Ltd.) were saturated with undiluted specific mycoplasma rabbit antiserum, dried at 37° C. and then stored at −20° C. until ready for use. The disks were placed on the inoculated agar surface, and the plates were incubated until the colonies became visible.

Antiserum production

Specific antiserum was prepared in rabbits against the 1836 strain of *M. bovigenitalium*. The organism was grown in broth, centrifuged at 10,000 rev./min. for 30 min., the supernatant fluid removed and the sediment resuspended in

physiological saline. The cells were washed six times and finally resuspended in physiological saline to a concentration equal to 2 × no. 10 Brown's opacity tube (Wellcome Research Laboratories). Rabbits were inoculated at weekly intervals for 6 weeks. Initially rabbits were inoculated subcutaneously at several sites with a total of 0·5 ml. antigen, homogenized in an equal amount of incomplete Freund adjuvant (Difco). The second and third inocula were similar but were given intramuscularly. The final three inocula consisted of antigen alone and were given intravenously. The rabbits were bled 1 week after the last injection.

Serological techniques

Stained serum plate agglutination (SA) test

A stained agglutination antigen was prepared using the 1836 strain. The organism was grown in broth and when sufficiently grown as judged by turbidity was centrifuged at 10,000 rev./min. The culture sediment was suspended in phosphate buffered saline (pH 7·0) containing merthiolate (1/10,000). The antigen was dispensed in glass tissue grinders and subsequently diluted to a concentration equal to 2 × no. 10 Brown's opacity tube. Rose Bengal was added to make a final concentration of 1/10,000, and the antigen was stored at 4° C. The SA test was carried out at room temperature; to one drop of serum on an enamel plate was added one drop of antigen; the two were mixed and the plate rotated. The test was read after 2 min., and a positive reaction was indicated by definite clumping. With positive reactors, twofold dilutions of the serum were prepared in physiological saline and the serum titres established.

Complement-fixation (CF) test

The CF test was used to detect the serological response in cows to *M. bovigenitalium*, strain 1836, and to determine whether a serological response occurred to *M. mycoides* antigen. The CF test of Campbell & Turner (1963) was used. Two 100% units of complement were used with *M. mycoides* antigen and $1\frac{1}{4}$ units with *M. bovigenitalium*. Serial twofold dilutions of serum commencing at 1/10 were used, and the titration end-point was the serum dilution in the dilution following complete fixation.

The 1836 antigen was prepared by growing the mycoplasma in broth, centrifuging, washing three times in physiological saline and resuspending in physiological saline to 3 × no. 10 Brown's opacity tube. The suspension was boiled for 15 min., and 4 vol. of ether were then added to 1 vol. of the concentrated organisms. The mixture was shaken vigorously for 5 min., the supernatant ether layer removed, and the remainder placed in a water-bath at 60° C. and shaken to remove last traces of ether. The *M. mycoides* antigen was the boiled antigen mentioned previously, which had no ether treatment.

The CF test was also used to investigate the relationship between *M. mycoides* and *M. bovigenitalium*. Specific rabbit antiserum to *M. mycoides* was supplied by Dr R. H. Leach, Mycoplasma Reference Laboratory, Colindale, London. In this test $1\frac{1}{4}$ units of complement were used with both antigens.

Separation of immunoglobulins by density gradient centrifugation

One ml. of serum, diluted 1/2 in phosphate buffered saline (pH 7·2), was layered on a four-step linear sucrose gradient (10, 20, 30 and 40%, w/v) and centrifuged for 16 hr. at 35,000 rev./min. in a Spinco ultracentrifuge, using the SW 39 swinging bucket rotor. Ten serial fractions were collected through a hole pierced in the bottom of the centrifuge tube, using an MSE fraction collector. Fractions 2–3 are associated with IgM (19 S) activity and fractions 6–7 associated with IgG (7 S) activity (Kunkel, 1960; Coghlan & Weir, 1967).

Treatment of sera with 2-mercaptoethanol

Sera were treated with an equal volume of 0·2 M mercaptoethanol and incubated at 37° C. for 1 hr. IgM (19 S) is usually mercaptoethanol sensitive and IgG (7 S) is usually mercaptoethanol-resistant (Deutsch & Morton, 1958).

Treatment of sera with rivanol

Three parts of a 0·4% solution of rivanol (2-ethoxy, 6,9-diaminoacridine lactate) were added to one part of serum. The mixture was left at room temperature for 15 min., then centrifuged at 2000 rev./min. for 10 min. The supernatant was removed and tested serologically. Rivanol precipitates all the serum proteins except the γ-globulins (Patras & Stone, 1961).

RESULTS

Mastitis was produced in all the cows infected via the udder with clinical signs appearing within 24 hr. No clinical response was seen in the animals inoculated via the vagina and via the nasal cavity. The infected quarters became swollen and tender, while the uninfected quarters remained unchanged. The clinical signs and secretions of the affected quarters were characteristic of *M. bovigenitalium* mastitis (Stuart *et al.* 1963; Afshar, Stuart & Huck, 1966). By the 17th day after inoculation, the infected quarters of cows C and D had recovered from the infection and the appearance of the milk was normal. The left hind quarters of cows A and B did not recover and by the 19th week the quarters were dry. On the 18th day after inoculation, cow A was found to be very lame. The left hind stifle joint was found to be very painful and severely swollen, with the swelling extending up and down the limb. By the 20th day after inoculation the left carpal joint also was severely swollen; fluid was removed aseptically from the carpal joint and cultured for mycoplasma and other bacteria (Cowan & Steel, 1965). *Bacillus cereus* was isolated.

Milk samples were taken from the four cows for 18 weeks. *M. bovigenitalium*, strain 1836, was always isolated from the milk of the affected quarters. The mycoplasma could be isolated quite readily by culturing directly on PPLO agar. When mycoplasma colonies were not evident by this method, they were always isolated using sloppy agar medium. Mycoplasma were not isolated from the blood samples taken from the cows in the first 3 weeks after infection. *Bacillus*

cereus was, however, isolated from the blood on the ninth and eleventh days after inoculation. The *B. cereus* was isolated by way of the sloppy agar cultures.

The antibody response in three of the cows is shown graphically in Fig. 1. Cows A–D are the cows inoculated via the teat canal, the response in cow D was

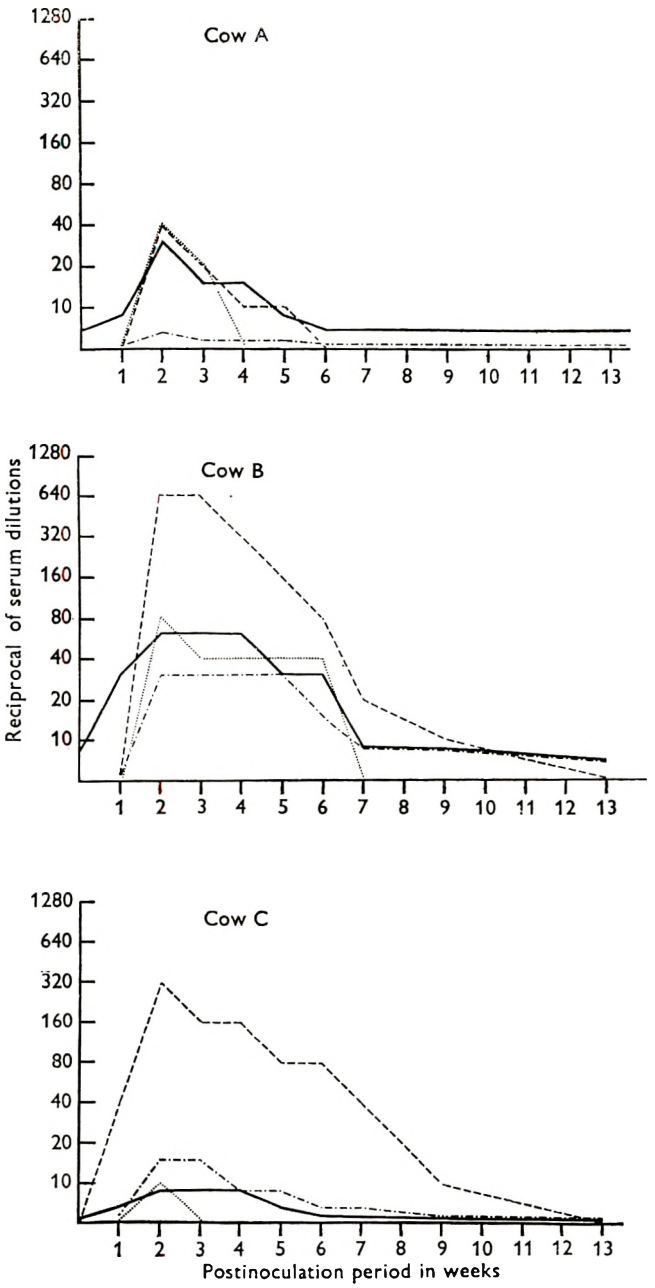

Fig. 1. Serological response in cows A, B and C to *M. bovigenitalium* strain 1836. — — —, 1836 CF reaction; · · · · · ·, CBPP CF reaction; ———, antiglobulin reaction; — · —, 1836 SA reaction.

similar to that seen in cow C. The two cows inoculated via the vagina and nasal cavity did not produce any detectable antibody response as indicated by the CF and SA tests.

By the 5th day after inoculation an antibody response was detected in the two cows C and D using the SA test, although in cow D the reaction was delayed. By the 6th day, all the cows gave a positive reaction, but in cow B the reaction was again delayed, with 5 min. elapsing before clumping occurred. By the 7th day all four cows gave positive SA reactions, with the reactions occurring within 2 min. The highest SA titres observed in the cows varied from 1/4 in cows A and D to 1/16 in cow C and 1/32 in cow B; these titres occurred 2 weeks after infection and were followed by a gradual decline. The SA reaction appeared to be specific for *M. bovigenitalium*, strain 1836, infection. Blood samples taken from the laboratory herd were tested: only one serum sample out of a total of 58 gave a positive reaction. It was later found that this cow had been infected 6 years previously via the teat canal with strain 1836 (Stuart *et al.* 1963). Serum from this cow did not react with *M. mycoides* antigen in the CF test.

The homologous antibody response to *M. bovigenitalium*, as measured by the CF test, varied in the four cows. Maximum titres were reached 2 weeks after inoculation, and then the titres declined, and were less than 1/10 by the 6th week in cow A, by the 7th week in cow D, and by the 13th week in cows B and C.

The heterologous antibody response to *M. mycoides* varied considerably in the four cows, as did the anti-γ-globulin response. The maximum heterologous titres varied from 1/10 to 1/80 in the four cows; these titres were attained 2 weeks after inoculation. By the 3rd week after inoculation the titres were less than 1/10 in cows C and D. Titres greater than 1/10 persisted until the 4th week after inoculation in cow A and until the 7th week after inoculation in cow B. All four cows showed an anti-γ-globulin response as a result of inoculation with *M. bovigenitalium*. The anti-γ-globulin response followed a pattern similar to that of the heterologous antibody response to *M. mycoides* and the two appeared to be related. A titre of 1/64 was the highest anti-γ-globulin titre observed and this occurred in cow B, the cow that gave the highest heterologous antibody response to *M. mycoides* antigen. The lowest anti-γ-globulin titre of 1/8 occurred in cows C and D, the cows that also gave the lowest heterologous antibody responses to *M. mycoides* antigen.

With serum samples from cow B, collected on the 2nd, 3rd, 4th, 5th and 6th weeks after infection, only partial fixation occurred at the 1/10 and sometimes at the 1/20 serum dilution, in the CF test when titrated against 1836 antigen. During this period, high anti-γ-globulin titres were observed in the serum samples.

The rabbit antisera to *M. mycoides* and *M. bovigenitalium* were titrated for complement-fixing ability against *M. mycoides* and *M. bovigenitalium* antigens. There was no evidence of cross-reactivity among antigens and antisera.

The bovine and rabbit sera were subjected to sucrose density gradient centrifugation. In the serum samples collected from the four cows inoculated with the 1836 strain, all the homologous and heterologous antibody activity was detected

in the 19 S fractions. These included the SA reaction, anti-γ-globulin reactions, the CF heterologous reaction against *M. mycoides* antigen and the CF homologous reaction against *M. bovigenitalium* antigen. The treatment of the same sera with mercaptoethanol and rivanol confirmed these results. The SA and anti-γ-globulin reactions were sensitive to mercaptoethanol treatment. Treatment of the sera with rivanol removed the SA, anti-γ-globulin, CF homologous and CF heterologous activities. These results differed from those obtained with serum samples taken 2 years previously from a cow, 3 weeks after infection with the M 120 strain of *M. bovigenitalium* (Afshar *et al.* 1966): antibody activity was found both in the 7 S and 19 S fractions, with most of the activity in the 7 S fraction. The rivanol and mercaptoethanol treatments confirmed these results.

In the CBPP positive bovine serum and in the *M. bovigenitalium*, strain 1836, rabbit serum, antibody activity was associated with both the 19 S and 7 S fraction, with most of the activity associated with the 19 S fraction. The results were confirmed with mercaptoethanol and rivanol treatments.

DISCUSSION

A feature of the antibody response to the 1836 strain of *M. bovigenitalium* was the anti-γ-globulin response in the serum of the four cows infected via the udder. The response varied in the four cows, with the highest titre occurring in the cow which had the highest titre before inoculation. The results indicate that the anti-γ-globulin response was responsible for the heterologous response to *M. mycoides* antigen. Mycoplasma was not recovered from the blood during the three postinoculation weeks, nor was it recovered from the swollen joints of cow A. Lameness has been a feature of mycoplasma mastitis and mycoplasmas have been recovered from the joints (Jasper, Jain & Brazil, 1966). The significance of the *B. cereus* isolation from the blood and joints is not known, but *B. licheniformis* has been isolated from infectious synovitis in chickens (Roberts, 1964) and rod-shaped bodies have been seen in rheumatoid synovia (Roy & Ghadially, 1967). The high anti-γ-globulin activity in cow B may also have been responsible for the procomplementary activity of the serum.

M. bovigenitalium persisted in the udder for the duration of the experiment, which was 18 weeks. Latent infection is usually associated with persistence of serum antibodies. The maximum titres were reached 2 weeks after infection and then declined. The CF titres were less than 1/10 by the 13th week. The rapid decline in CF titres was striking, especially as the organism was still easily isolated from the milk. The CF test used in this experiment was not sensitive enough to detect low titres, but antibodies were detected using the SA test. The SA test was specific and was able to detect cows that were infected with the same organism 6 years previously.

In man and rabbits following immunization or infection with *M. pneumoniae*, the antibodies consisted of 19 S and 7 S (Schmidt, Lennette, Dennis & Gee, 1966; Fernald, Clyde & Denny, 1967). A factor which influences the kind of antibody produced is the nature of the antigen; lipopolysaccharide somatic antigens of entero-

bacteria stimulate IgM antibodies predominantly in man and rabbit (Pike, 1967). The inoculation of cows with *M. bovigenitalium*, strain 1836, produced IgM antibodies, whereas with the M 120 strain IgG was the predominant antibody. The CF test indicated that the two strains were similar. The two antigens used in the CF test were prepared differently, the 1836 strain had to be treated with ether before it was suitable, whereas the antigen of the M 120 strain was a washed suspension of the organism. The two strains appear to be antigenically distinct when compared using the growth inhibition test. These results are in agreement with those of Leach (1967), although he suggests that the use of a more potent antiserum would have shown an antigenic relationship between these strains by the growth inhibition test.

SUMMARY

Four cows were inoculated via the udder with the 1836 strain of *Mycoplasma bovigenitalium*. The serological response was investigated using the complement-fixation and serum plate agglutination tests. A heterologous response to *M. mycoides* was observed, associated with an increase in the anti-γ-globulin titres in the cow's serum. The heterologous response persisted in one cow for 6 weeks after infection. The nature of the immunoglobulin response was characterized by density gradient centrifugation, and by mercaptoethanol and rivanol treatment of the serum and was found to consist entirely of 19 S γ-globulins.

The *Mycoplasma* persisted in the udder for the 18 weeks duration of the experiment. The *Mycoplasma* was not isolated from the blood. One of the cows became lame with swollen joints; *Bacillus cereus* was isolated from the joints and blood.

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Attempts to recover *Mycoplasma suipneumoniae* from experimental and natural cases of enzootic pneumonia in pigs

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Goodwin & Whittlestone (1964, 1966) induced enzootic pneumonia in pigs with a mycoplasma-like agent grown in liquid medium. Using essentially this medium, Maré & Switzer (1965) also reproduced enzootic pneumonia with liquid-medium cultures, and from these cultures they isolated a mycoplasma on solid medium, which they called *Mycoplasma hyopneumoniae*. However, at least one mycoplasma other than the causal agent of enzootic pneumonia commonly occurs in the respiratory tract of pigs affected with enzootic pneumonia and it cannot be assumed, therefore, that a mycoplasma isolated on solid medium is the same as a pneumonia-inducing agent grown in parallel in liquid medium. Goodwin, Pomeroy & Whittlestone (1965) proved that the mycoplasma isolated from their pneumonia-inducing fluids was indeed the causal agent of enzootic pneumonia by passaging it serially on solid medium until a dilution of at least 10^{-15} of the primary seed inoculum was reached; these final colonies induced enzootic pneumonia. This mycoplasma was named *Mycoplasma suipneumoniae*. Subsequently, *M. suipneumoniae* was shown to be serologically different from a wide range of other mycoplasmas (Goodwin, Pomeroy & Whittlestone, 1967), and hence it is probably a new species.

The relationship and nomenclature of these porcine mycoplasmas is complicated by the fact that the culture distributed as *M. hyopneumoniae* did not derive from the colonies on solid medium that were named *M. hyopneumoniae* by Maré & Switzer (1965), but from their pneumonia-inducing fluids (W. P. Switzer, 1968, personal communication). The cross-neutralization between *M. suipneumoniae* and *M. hyopneumoniae* in the growth-inhibition and metabolic-inhibition tests, therefore, shown by Goodwin *et al.* (1967), indicates a relationship between *M. suipneumoniae* and a mycoplasma derived from pneumonia-inducing fluids in the United States, and not between *M. suipneumoniae* and the colonies originally published as *M. hyopneumoniae*. Because of this difficulty, and because the name *M. suipneumoniae* refers to a mycoplasma of known pathogenicity, which has been compared with other mycoplasmas serologically, we are continuing to use this name. A precise link then exists with our previous work, and this should prevent further confusion.

Enzootic pneumonia of pigs appears from clinical evidence to be a widespread disease, both in this country and abroad. Hitherto, however, because there has been no precise method of diagnosis, the limits and distribution of the disease

have not been defined. Clearly, the isolation of the causal agent offers a considerable advantage in this respect, especially when there is no published information on the diagnostic value of specific antibodies in pig sera. This paper presents a preliminary assessment of the extent to which the isolation of *M. suipneumoniae* might be routinely possible.

Table 1. *Attempts to isolate* Mycoplasma suipneumoniae *from experimentally induced cases of enzootic pneumonia*

Pig	Type*	Strain of experimental infection	Isolation of mycoplasmas: on solid medium	Isolation of mycoplasmas: in liquid medium	Serological identification
2856	HPCD	J	–	+	
2857	HPCD	J	–	+	
2860	HPCD	J	–	+	
2878	HPCD	J	–	+	
2958	HPCD	J	–	+	
2959	HPCD	J	–	+	
2895	HPCD	J	–	+	
2896	HPCD	J	–	+	
2805	HPCD	J	–	+	
3012	HPCD	J	ND	+	*M. suipneumoniae*
3013	HPCD	J	ND	+	
3014	HPCD	J	ND	+	
2912	MH	CZ	–	+	
2915	HPCD	CZ	–	+	
2922	MH	CZ	–	+	
2923	MH	CZ	–	+	
2897	HPCD	CZ	–	+	
2910	HPCD	CZ	–	+	
2911	HPCD	CZ	–	+	
2913	MH	CZ	–	+	
2932	HPCD	CZ	–	–	Not applicable
2898	HPCD	CZ	–	–	

* See Materials and Methods (p. 599) for definitions.
ND = not done.

MATERIALS AND METHODS

Pneumonic-lung samples

The two strains (J and CZ) of *M. suipneumoniae* that were in the lung samples used to induce the cases of enzootic pneumonia shown in Table 1 originated from two adult sows in separate herds; both these cases of enzootic pneumonia were chosen because, when first studied, the lung lesions did not yield any readily cultivable mycoplasmas. For further information on the history of the J strain, see Goodwin & Whittlestone (1963). The CZ strain has been cultured repeatedly in the laboratory (in tissue cultures, in liquid media and on solid media) and re-isolated from transmission experiments in pigs, without the appearance of any mycoplasma other than *M. suipneumoniae*; it is specifically neutralized in the metabolic-inhibition and growth-inhibition tests by serum prepared against the J strain of *M. suipneumoniae*.

The field strains listed in Table 2 all came from sudden outbreaks of enzootic pneumonia which arose in 12 separate herds that had been in a control scheme for herds believed to be free from this disease (Goodwin & Whittlestone, 1967). These outbreaks had the clinical and epidemiological characteristics of enzootic pneumonia (Goodwin & Whittlestone, 1967), and the pneumonic cases used satisfied the pathological and touch-preparation criteria that are referenced later. Pigs 2750, 2645, 2644, 2901, 2902, 2903 and 603 were specially killed, in order to harvest lung specimens aseptically; all the other pigs in Table 2 were killed at slaughterhouses, and the lesions were sampled after the lungs had been removed in the routine way on the slaughter line.

Lung samples that were not cultured when fresh were stored at −30° C. or −60° C.

Table 2. *Attempts to isolate* Mycoplasma suipneumoniae *from field cases of enzootic pneumonia*

Herd of origin	Pig	Isolation of mycoplasmas: on solid medium	Isolation of mycoplasmas: in liquid medium	Serological identification
EO	9952	−	+	*M. hyorhinis*
	9954	−	+	
HU	9949	−	+	*M. suipneumoniae*
	9948	−	+	
IT	9920	−	+	*M. hyorhinis*
	9921	+	+	
US	9906	−	+	*M. hyorhinis*
	9909	−	+	*M. suipneumoniae*
TZ	9902	−	−	NA
	9901	−	+	*M. hyorhinis*
CL	9769	−	−	NA
	9770	−	+	*M. hyorhinis*
TE	2750	−	+	*M. hyorhinis*
	2752	−	−	NA
XI	2645	−	−	NA
	2644	−	−	
TP	2901	−	+	*M. hyorhinis*
	2902	−	+	
	2903	−	+	
CO	9960	+	+	*M. hyorhinis*
	9962	−	−	NA
PF	2982	−	+	*M. hyorhinis*
	2983	−	−	NA
MG	603	−	ND*	*M. hyorhinis**

* This isolation was made by a different method (see Materials and Methods p. 599).
ND = not done; NA = not applicable.

Disease-transmission experiments in pigs

Two types of pig were used: hysterectomy-produced, colostrum-deprived (HPCD) animals, which had been reared in strict isolation, and naturally born pigs (MH) from a herd established only from HPCD pigs; the lungs of routinely slaughtered pigs from this herd are regularly checked for the absence of enzootic pneumonia. Apart from the first passage from herd MG (Table 3), which was made in ordinary isolation accommodation, all the disease-transmission experiments

Table 3. *Disease-transmission experiments with six field strains of enzootic pneumonia*

Herd of origin	Material providing inoculum	Experimental inoculations: Pigs	Experimental inoculations: Presence of enzootic pneumonia	Recovery of mycoplasmas: on solid medium	Recovery of mycoplasmas: in liquid medium	Serological identification
EO	9952, 9954	2994	+	−	+	*M. suipneumoniae*
		2995	+	−	+	
	Controls for EO and IT	2992	−	.	.	.
		2993	−	.	.	.
IT	9920, 9921	2996	+	−	+	*M. hyorhinis*
TZ	9901, 9902	2873	+	ND	−	NA
		2874	+	ND	−	
	Control	2875	−	.	.	.
	2873, 2874	2925	+	−	+	*M. suipneumoniae*
		2926	+	ND	ND	
	Control	2927	−	.	.	.
XI	2644, 2645	2658, 2661, 2663	+	−	−	NA
	Control	2660	−	.	.	.
TP	2900, 2901, 2902, 2903	2975	+	+	+	*M. hyorhinis*
MG	603	2029*	+	−	ND	.
		2039*	+	−		
		2044*	+	ND		
	Controls	162*, 163*, 164*	−	.	.	.
	2029, 2039	2264	+	−	ND	.
	2264	2321	+	−	ND	.
		2324	+	−	ND	*M. suipneumoniae*
	Control	2322	−	.	.	.

ND = not done; NA = not applicable.
* These six pigs were type MH: all the others in this Table were HPCD.
† This isolation was made by a different method (see Materials and Methods).
Note. No attempts were made to recover mycoplasmas from the control pigs.

shown in the tables were made in a specially designed building: each cubicle is approached through an ante-room in which protective clothing is put on, and before each experiment all the equipment in the cubicles is first steamed and then fumigated with formalin within the cubicle itself.

Apart from pigs 2029, 2039 and 2044 in Table 3, which received 20 ml. each of a 10^{-2} lung suspension intra-tracheally, all the experimentally infected pigs in Tables 1 and 3 were inoculated intranasally with 4–30 ml. of a 1/25 to 1/4 suspension of ground pneumonic lung in broth.

All the lung samples from the experimentally infected pigs were harvested aseptically, after removal of the sternum.

The initial laboratory diagnosis of all the cases of enzootic pneumonia mentioned in this paper was based on the nature of the gross lesions, the histological picture and the examination of touch preparations for organisms with the morphology of *M. suipneumoniae*, as described elsewhere (Goodwin & Whittlestone, 1966).

Cultural examinations

When studying the outbreaks of enzootic pneumonia listed in Table 2, apart from herd MG, samples of pneumonic lesions from several pigs in each outbreak were usually available. The general procedure was to select first a case where the stained touch preparations made from the lesions showed many mycoplasmas morphologically similar to *M. suipneumoniae*: if this case did not yield a mycoplasma on culture, or if *Mycoplasma hyorhinis* was cultured, the next best case (as judged by touch preparations) was then examined. To this extent, therefore, the lesions cultured from the field were not random samples.

Media

Except for pig 603 (Table 2) and pig 2324 (Table 3), the liquid and solid media used contained Hartley's broth, and were prepared and incubated as previously described (Goodwin *et al.* 1967). Mycoplasma 603 was isolated first in pig-lung monolayer cultures, from which it was passaged via Edward's medium to pig-kidney monolayer cultures and then to solid medium containing Hartley's broth; after five single-colony passages on this solid medium, it was grown in the Hartley's-broth liquid medium. Mycoplasma 2324 was initially isolated in plasma-clot cultures prepared from the freshly harvested pneumonic lesions; it was then passaged to pig-lung monolayer cultures and subsequently to boiled-tissue-culture medium (Goodwin & Whittlestone, 1966), from which it was passaged to liquid medium for identification.

The solid medium used for the cultural examinations relating to herd MG in Tables 2 and 3 was serum-brucella-agar medium described elsewhere (Goodwin & Whittlestone, 1962).

Inoculations and assessments

Fresh lung, or rapidly thawed lung from the deep freeze, was ground in a Griffith's tube with liquid medium, to make a 1/10 dilution. Further dilutions were then made in liquid medium, usually to give final lung dilutions of 1/200–

1/2000 and, before incubating these, samples from them were seeded on to solid medium.

Growth in liquid medium was assessed by the production of acid but, before carrying out a metabolic-inhibition test, the acid-producing agent was passaged two or three times.

In several instances, mycoplasma-type colonies were isolated directly on solid medium but these isolations are not noted in the text and tables, unless the colonies could be passaged on solid medium.

Serological techniques

The growth-inhibition test of Huijsmans-Evers & Ruys (1956), as modified by Clyde (1964) and Stanbridge & Hayflick (1967), was used: the disks were soaked in 0·025 ml. of antiserum, allowed to dry, and stored at −20° C. until required. The metabolic-inhibition tests (Taylor-Robinson, Purcell, Wong & Chanock, 1966), using either microtitre plastic plates or small glass tubes, were made with rabbit antiserum R 1 against *M. hyorhinis*, or R 2 against *M. suipneumoniae* (Goodwin *et al.* 1967); medium and organism controls were included with each test.

RESULTS

Experimental cases of enzootic pneumonia

Twenty-two cases of experimentally induced enzootic pneumonia were cultured for mycoplasmas; these cases were unselected and all the examinations made during a certain period of time are included. The results are shown in Table 1.

In no instance was a primary isolation made directly on solid medium. In 20 of the 22 cases, however, a mycoplasma was cultured in liquid medium, and in all of these cases the mycoplasma was identified as *M. suipneumoniae*.

Field cases of enzootic pneumonia

After finding that *M. suipneumoniae* could be cultured from a high percentage of experimentally induced cases of enzootic pneumonia, 11 field outbreaks of the disease were studied in the same way. These results are shown in Table 2, together with a twelfth outbreak (strain MG); this outbreak, which is included because it provided relevant results in disease-transmission experiments (see Table 3) and because it yielded our working strain (603) of *M. hyorhinis*, had been studied earlier by a different method, in 1961.

It can be seen that in only two of the 12 outbreaks was *M. suipneumoniae* isolated. In seven of the total number of 24 pigs examined, no mycoplasma was recovered, and with herd XI both pneumonic cases came into this category; this outbreak will be referred to again in the next section (disease-transmission experiments). In nine of the 12 outbreaks, the only mycoplasma identified was *M. hyorhinis* and the question arose, therefore, whether these nine outbreaks were truly cases of enzootic pneumonia, even though they simulated the disease in other respects. To throw some light on this question, material from six of the outbreaks that had not yielded *M. suipneumoniae* was used in disease-transmission experiments.

Disease-transmission experiments

The six herd outbreaks of enzootic pneumonia investigated in this way comprised five of the 11 recent outbreaks, plus outbreak MG from 1961. The results are shown in Table 3.

In the first passage of the disease from herd TZ, the areas of induced pneumonia were extremely small in both pigs. A second passage was made, therefore, and as the lesions in pig 2925 in this passage were much more extensive than in pig 2926, the former lesions were chosen for cultural examination.

The pig inoculum for the second passage from herd MG was clarified through a Seitz no. 5 pad and then taken through a Millipore filter (APD 0·8 μ).

Table 3 shows that the lesions from herd XI, from which no mycoplasmas had been cultured, transmitted as enzootic pneumonia, as did the lesions from all the other five outbreaks, which had been complicated originally by *M. hyorhinis*. Two of the latter five transmissions (IT and TP) continued to yield *M. hyorhinis* from the first-passage pneumonias, but the remaining three transmissions yielded *M. suipneumoniae* at the first (EO), second (TZ) and third (MG) passages, respectively.

DISCUSSION

In 10 of the 12 outbreaks listed in Table 2, a diagnosis of enzootic pneumonia could not be reached by isolating and identifying *M. suipneumoniae*. Six of these 10 outbreaks, however, were selected at random and lung lesions from each of them readily induced a disease indistinguishable from enzootic pneumonia in animal transmission experiments. It seems very probable, therefore, that the remaining four herd outbreaks, which had the same features as the previous six, were also due to enzootic pneumonia. If this assumption is correct, the failure to make a diagnosis in 10 out of 12 outbreaks was due in one instance to the inability to isolate any mycoplasmas, and in nine instances to the simultaneous presence of *M. hyorhinis*. In our experience, therefore, *M. hyorhinis* is commonly present in field cases of enzootic pneumonia and hence the isolation of an unidentified mycoplasma is of no value in the diagnosis of this disease, particularly when the presence of *M. hyorhinis* appears to prevent the isolation of *M. suipneumoniae*. Conversely, when *M. hyorhinis* appeared to be absent, *M. suipneumoniae* was isolated in 24 out of 31 experimental cases, and three out of 10 field cases, making a total of 27 out of 41 cases, or 66 %. In the 22 experimental transmissions using the J or CZ strains (Table 1), the isolation rate of *M. suipneumoniae* was 91 %.

M. suipneumoniae was not isolated directly on solid medium in any of the 26 experimental and field cases where it was isolated in liquid medium, and there could be several reasons for this. Perhaps either the present solid medium or the way it is used is unsuitable for such direct isolation. On the other hand, there could be inhibiting factors in the lung which could still present a problem, even with an improved medium or better cultural methods. If a way could be found of isolating *M. suipneumoniae* directly on solid medium, without the intervention of liquid medium, it should be possible to distinguish it, even if it were mixed with *M. hyorhinis* or other mycoplasmas.

It is interesting that in three of the five transmission experiments in pigs where the presence of *M. hyorhinis* was an initial complication, this mycoplasma disappeared from the lung lesions on passage. It may be that in the early stages of the disease at least, the pig—when inoculated intranasally—is able to eliminate *M. hyorhinis* or confine this mycoplasma to the upper respiratory tract. In this sense, the live pig can be used as a selective medium, but in our limited experience the failure rate is high (at least 40 % on first passage); the method is also expensive and, clearly, a simple and much more uniformly reliable selective laboratory medium would be very helpful at this stage.

SUMMARY

Two strains of enzootic pneumonia, which are regularly free from mycoplasmas other than *Mycoplasma suipneumoniae*, were transmitted experimentally to a total of 22 pigs. *M. suipneumoniae* was recovered in liquid medium from the lung lesions in 20 of these animals (91 %).

Twelve field outbreaks of enzootic pneumonia were likewise examined for *M. suipneumoniae*: from each outbreak, cases were selected where many mycoplasmas with the morphology of *M. suipneumoniae* were seen in touch preparations made from the lung lesions. *M. suipneumoniae* was recovered from only two of these outbreaks, but *M. hyorhinis* was obtained from nine of them.

To establish whether the outbreaks yielding only *M. hyorhinis* were indeed enzootic pneumonia, material from five of them was used in disease-transmission experiments: enzootic pneumonia was induced in every instance, and *M. suipneumoniae* was recovered from three of the five transmitted strains.

It is concluded that *M. hyorhinis* is commonly present in the lung lesions of enzootic pneumonia and that this mycoplasma may often prevent the isolation of *M. suipneumoniae*. The problem of recovering *M. suipneumoniae* in this situation is made more difficult by the fact that in no instance where *M. suipneumoniae* was isolated directly in liquid medium was it cultured in parallel on solid medium.

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An alternative method of phage-typing *Staphylococcus aureus**

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INTRODUCTION

The present internationally agreed system for the phage-typing of *Staphylococcus aureus* (Blair & Williams, 1961; Report, 1967) is derived from the method of Wilson & Atkinson (1945). A plate of nutrient agar is flooded with a 6 hr. broth culture of the staphylococcus, and a drop of each of the basic set of 22 phages, diluted to the routine test dilution (RTD), is placed on the plate. The RTD is defined as that dilution of phage which produces near-confluent lysis on the propagating strain. If none of the phages produces lysis to the extent of 50 plaques or more, the culture is retyped on the following day with the same phages at 1000 times the routine test dilution (RTD × 1000). Williams & Rippon (1952) found that, when typing is carried out at RTD, less than 5% of epidemiologically related sets of cultures showed differences of typing pattern of more than one strong phage reaction (i.e. no lysis of one culture and a reaction exceeding 50 plaques with another). No similar investigation of the variation in typing patterns with phages at RTD × 1000 has been reported, but there is evidence that reproducibility is relatively poor, either because of confusion between lysis and inhibition reactions or because of the frequency with which staphylococci are lysed by many phages at this dilution (Pöhn, 1957).

It seemed, therefore, that although the use of the phages at RTD × 1000 increased the proportion of typable staphylococci, it might add little information about the relation of one culture to another. An investigation was therefore carried out to see if, by making additions to the present basic set of phages, it would be possible to type an acceptably high percentage of staphylococci at RTD and so to remove the necessity to use phages at RTD × 1000. A large number of phages from the collection maintained in this laboratory were examined, and 20 phages were eventually chosen for use, together with the phages of the present basic set, for typing at RTD. A comparison was made of the reproducibility of the individual reactions and of the patterns produced by the basic-set phages and the extra phages.

* The contents of this paper form part of a thesis submitted for the degree of Doctor of Medicine in the University of Oxford.

MATERIALS AND METHODS

Propagating strains

Freeze-dried preparations of the propagating strains for the phages were reconstituted in broth. Single colonies were picked and typed at RTD × 1000 with the present basic set of phages. RTD × 1000 was used since the original records indicated that typing of many of the propagating strains had been only with phages at that strength. Many of the propagating strains were untypable at RTD, and for these strains no useful comparison could be made of phage reactions except at RTD × 1000.

Typing method

Typing was performed by the method of Blair & Williams (1961). Dilutions of phage were placed in holes in a Perspex block and were applied to the surface of the agar plates with a multiple-loop applicator (Lidwell, 1959). Plastic Petri dishes were used containing a base layer of the following medium: 1 % Evans peptone, 0·5 % NaCl, 1·2 % shred agar. This was covered with a layer of a second medium: 2 % Difco no. 2 nutrient broth, 0·5 % NaCl, 1·2 % shred agar. Calcium chloride was added to a final concentration of 400 μg./ml.

Plates were read with a large hand-lens after overnight incubation at 30° C., and phage reactions were recorded as follows: ++ = 50 plaques or more, + = 20–49 plaques, ± = 10–19 plaques, ±1, ±2, ±3, . . ., ±9 = 1, 2, 3, . . ., 9 plaques.

Phages

Phages had been stored either as broth suspensions at 4° C. or freeze-dried. Some of those stored as broth suspensions were of adequate titre for the experiment. Freeze-dried phages were reconstituted in broth. When necessary, phages were propagated by the soft-agar layer method originally described by Hershey, Kalmanson & Bronfenbrenner (1943) and later modified by Swanstrom & Adams (1951), and were titrated by placing 0·02 ml. drops of tenfold dilutions on a lawn of the appropriate indicator strain.

In all, 119 staphylococcal phages other than those of the present basic set were available in the laboratory; 27 of them had at some time been rejected from the basic set or from the set of additional phages used between 1953 and 1962; the rest had been sent to the Staphylococcus Reference Laboratory as potentially useful phages for typing staphylococci. Phage P3, which was received from Mrs H. Landau of Belfast after the investigation had begun, was included at a later stage.

After a preliminary study, 20 phages were chosen for a more detailed investigation. The lytic ranges of these phages were determined in the same manner, and with the same set of test staphylococci, as is recommended for the basic-set phages (Blair & Williams, 1961). With a high-titre phage preparation, the ratio was found of the dilution of phage which gave a standard amount of lysis of each test strain to the dilution which gave the same degree of lysis of the propagating

strain. Phage suspensions were diluted from stock suspensions to RTD each week and stored at 4° C., and test dilutions were checked on the propagating strains before use.

For the purposes of the investigation, typability was taken as a phage reaction of + or more (20 plaques or more). When staphylococci were typed with the basic-set phages at RTD × 1000, no account was taken of inhibition reactions or of plaques in an area of inhibited growth (Blair & Williams, 1961).

Cultures of Staphylococcus aureus *typed*

Cultures of *Staph. aureus*, which had been received sequentially at the Staphylococcus Reference Laboratory, were used to test the usefulness of various phages for typing. Cultures from outside the British Isles, and from veterinary sources, were excluded. In all, 5000 cultures received during 1966 and 1967 were typed. The reproducibility of the typing results and of the ability of the different sets of phages to distinguish between staphylococcal cultures was investigated with 782 pairs of cultures which had been isolated from the same person in St Bartholomew's Hospital at an interval of 5–10 days. Antibiotic sensitivity tests had been carried out on these cultures.

RESULTS

Selection of the extra phages

It was impracticable to investigate more than a proportion of the 119 available phages, so a preliminary selection was made on the basis of the available records of the character and performance of the phages. For example, when several phages were known to have a similar host-specificity, only one was included. Phages of serological groups D, G and H were generally not chosen, because their lytic activity is usually too non-specific. If a phage had been exhaustively tested before and found to be of no value it was also discarded. Only one phage, KS6, splitting the 52, 52A, 80, 81 complex was included. A representative selection of phages found useful for typing bovine staphylococci (Davidson, 1961) was also included. Only one phage of lytic group II was added (phage 51), because on the whole those in the present basic set were considered to produce an adequate differentiation of the staphylococci in this group. No phages were used which lysed only coagulase-negative staphylococci, and highly specific phages were not tested.

Phages 7 and 3B had been discarded from the basic set in July 1966 (Report, 1967) after it had been found that they were the sole phage lysing a culture of *Staph. aureus* in only two out of 14,000 strains (E. H. Asheshov, personal communication). They were therefore not studied in the present investigation. Phage 1380, now designated phage 87 (Report, 1967), was not included, because a recent survey carried out in this laboratory had not revealed any organisms lysed by it. Phage D, isolated by Wallmark, was also not included because of its resemblance to phages 84 and 85. Fifty-eight phages were discarded in this manner, and the remaining sixty-one (Table 1) were used in the first experiment.

Three thousand sequential cultures submitted to the Staphylococcus Reference

Laboratory for typing were first examined. The number typable by the basic set of phages at RTD was 2279 (75·9 %; see Table 2). When the untypable cultures were tested with the same phages at RTD × 1000, a further 411 were typed, bringing the percentage typed to 89·7. The addition of the 61 extra phages at RTD to the basic set at RTD resulted in the typing of 436 more cultures, a total percentage typed of 90·5; but 431 of these 436 cultures were lysed by only 20 extra phages. Thus the use of a combined set of 43 phages at RTD, which would have been accommodated easily in two standard typing blocks, resulted in the typing of 90·3 % of the cultures.

Table 1. *Set of* 61 *phages examined for their usefulness in typing cultures of* Staph. aureus *untypable with the basic set at RTD*

Phages with numbers allotted by the International Subcommittee			Other phages	
			Laboratory no.	Origin
29A*	47D*	77A*	UC 13, UC 14	
31*	51*	78*	UC 16, UC 20	Hill
31A*	52B*	86* (UC 18)	L 948, L 971	Leyton
31B*	57*	102†	B 33, C 33, D 33	Colquhoun
42B*	58*	103†	304, 377, 756, 950	Milch
42C*	62*	105†	SA 446, SA 313	Purandare
42F*	69*	107†	812	Rische
44*	70*	108†	Y 22, 777	Sharpe
44A*	73*	111†	Rosa, Israel C	Sompolinsky
47A*	75A*	190	A, B, C, E, KS 6	Wallmark
47B*	75B*	191		
47C*	76*	192		

* Previously in the basic set, or in the set of additional phages.
† 'Bovine' phages, see Davidson (1961).

Table 2. *Typing of* 3000 *cultures of* Staph. aureus *by the basic set of phages at RTD and at RTD* × 1000, *and by the basic set together with additional phages at RTD*

(A culture was considered typable if a phage produced 20 plaques or more.)

Typable by	No.	Percentage
Basic set at RTD	2279	75·9
Basic set at RTD + basic set at RTD × 1000	2690	89·7
Basic set at RTD + 61 extra phages at RTD	2715	90·5
Basic set at RTD + 20 extra phages at RTD	2710	90·3
Total number examined	3000	100

Characters of the extra phages

The origins of the 20 'useful' extra phages were found from the records (Table 3). They included phages 44, 47A and 69 previously in the original basic set, phages 31, 52B, 75A and 75B originally amongst the additional phages between 1953 and 1962 and phage 86 (originally UC 18). All the 'bovine' phages (Davidson, 1961) are excluded. Meyer (1967) found that only a very small additional number of human staphylococci were lysed by group IV phages other than 42D. An

additional phage, P3, which was currently under investigation, was added to the 20 phages at this stage of the investigation.

The typing patterns of the propagating strains of these extra phages with the present basic set at RTD × 1000 were investigated (Table 4). These patterns were similar to those recorded when the staphylococci were first examined in the laboratory.

Table 3. *Origins of phages used in the extra typing set capable of typing cultures of* Staph. aureus *which were untypable by the basic set at RTD*

Phage	Lytic group	Serological group	Origin	Date	Ref.
190	Misc.	B	Wahl	1954	.
191	Misc. and I	B	Wahl	1954	.
192	III	A	Wahl	1955	.
C 33	Misc.	B	Colquhoun	1958	.
D 33	?	B	Colquhoun	1958	.
Rosa	Misc.	B	Sompolinsky	1960	.
SA 446	I	B	Purandare	1960	.
69	I	B	Hood	1951	2
L 971	III	?	Leyton	1953	.
KS 6	I	A	Wallmark	1961	3, 5
UC 13	III	B	Hill	1963	.
86 (UC 18)	III	B	Hill	1963	6
E	Misc.	B	Wallmark	1962	4
C	III	F	Wallmark	1962	4
75B	III	A	Hood	1951	.
31	I	B	Wilson & Atkinson	1943	1
44	I	B	Wilson & Atkinson	1943	1
52B	III	B	Heimer	1948	2
47A	Misc.	A	Wilson & Atkinson	1943	.
75A	III	A	Hood	1951	.
P 3	III	B	Landau	1967	.

Figures in italic indicate internationally agreed phage numbers.

References: (1) Wilson & Atkinson (1945); (2) Williams, Rippon & Dowsett (1953); (3) Comtois (1960); (4) Wallmark & Finland (1961); (5) Wallmark (1954); (6) Thomas, Hill, Culbertson & Altimeier (1960).

Finally the 20 extra phages and P3 were characterized by their lytic ranges (Table 5). These lytic ranges are those found by the author, and are based on two separate estimations for each phage. The lytic ranges generally resembled those found in the earlier records. The few major changes do not, however, invalidate the experiment. Where these changes have occurred it must be noted that the phage had probably undergone some change either by mutation, host-induced modification or contamination.

Assessment of redundancy of the phages in the new typing set

The extra phages had been used in the first part of the investigation as a secondary typing system for cultures of staphylococci untypable by the basic set of phages at RTD. They were now used together with the basic set at RTD to obtain an estimate of the frequency with which reactions with the new phages

Table 4. *Typing patterns of the propagating strains of* 20 *extra phages with the basic set of phages at RTD* × 1000

Phage	Propagating strain	Lysis by phage: 29	52	52A	79	80	3A	3C	55	71	6	42E	47	53	54	75	77	81	42D	187
190, 192	854	.	++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Cl	.	.
191	838	Non-typable																		
C 33, D 33	33	++	++	++	+	Cl	.	.	.	.	.	++	Cl	Cl	++	Cl	++	++	.	.
Rosa	Rosa	.	.	.	.	.	.	.	.	.	.	.	.	Cl	.	Cl	.	.	.	.
SA 446	446	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	Cl	++	.
69	8397	.	++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.
L 971	8329	.	.	.	.	.	.	.	.	.	.	.	Cl	Cl	Cl	Cl	Cl	.	.	.
KS 6	KS 6	.	Cl	Cl	+	Cl	.	.	.	.	.	.	.	.	.	.	.	.	.	.
UC 13	UC 13	.	.	.	.	.	.	.	++	.	Cl	++	Cl	Cl	++	+	.	++	.	.
86	86	.	.	.	.	.	.	.	.	.	.	.	0	0	0	0	in	.	.	.
E	E	.	.	.	.	.	.	.	.	.	++	++	++	++	++	++	.	++	.	.
C	C	.	.	.	+	.	.	.	.	.	.	++	.	Cl	.	.	++	.	.	.
75B	9309	.	.	.	.	.	.	.	.	.	.	.	GR/Cl	.	.	Cl	.	in	.	.
31, 44	8508	Cl	Cl	Cl	Cl	Cl	.	.	.	±	.	*Cl*	Cl	*Cl*	Cl	++	*Cl*	Cl	*Cl*	.
52B	9303	.	+	.	.	.	.	.	+	.	*Cl*	++	Cl	Cl	*Cl*	.	*Cl*	++	+	.
47A	8317	+	.	.	.	±	.	.	.	.	.	.	.	.	.	.	.	.	.	.
75A	9307	.	±	.	.	.	.	.	.	.	.	.	.	.	.	++	.	.	.	.

± = less than 20 plaques; + = 20 to 49 plaques; ++ = 50 plaques or more; Cl = confluent lysis; *Cl* = not quite confluent lysis; 0 = inhibition; in = weak inhibition; **++** = lysis in an area of inhibition; GR/Cl = confluent lysis with resistant growth.

Propagating strains with four-figure numbers indicates that they are lodged with the National Collection of Type Cultures.

Table 5. *Lytic ranges of the* 20 *extra phages and phage* P3

	190	191	192	C 33	D 33	Rosa	SA 446	69	L 971	KS 6	UC 13	86	E	C	75B	31	44	52B	47A	75A	P 3
3A	.	.	.	2	2	.	.	.	.	.	.	.	.	.	.	2	1	1	.	.	2
3B	.	1	.	.	.	.	1	.	.	.	.	.	.	1	.	1	.	.	.	1	2
3C	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	2	.	1	1	1	0
6	4	4	4	.	3	4	4	3	5	4	4	5	3	5	5	0	.	4	1	5	5
7	4	4	3	.	3	4	3	3	4	3	4	5	3	5	5	.	.	5	.	3	> 5
29	4	.	.	.	.	4	.	3	.	4	.	1	.	0	.	4	.	.	.	.	.
29A	4	3	3	.	.	3	3	3	2	3	.	.	0	4	3	4	.	4	.	.	4
31/44	4	3	3	4	3	4	4	3	3	3	3	3	5	3	3	5	5	4	.	3	4
42B/47C	5	3	5	2	2	3	4	.	2	5	.	1	1	0	2	0	.	2	.	.	1
42C	4	4	.	4	.	4	4	.	2	.	3	4	.	4	2	.	.	0	.	3	4
42E	4	4	3	0	3	0	2	0	.	3	3	4	3	3	0	0	.	4	.	.	0
44A	4	4	3	.	.	0	4	3	3	3	.	.	2	0	0	0	1	2	3	0	0
47	2	0	.	.	3	3	3	1	2	2	4	.	2	3	5	3	1	5	1	4	> 5
47B	2	.	.	.	.	0	.	1	.	.	.	2	.	2	2	0	.	0	.	2	> 5
52	3	1	.	.	.	.	0	.	.	0	.	.	0	0	.	0	.	4	.	.	0
52A/79	3	5	.	3	2	0	2	.	.	.	.	1	1	0	.	0	.	4	.	.	0
53	0	.	.	.	3	4	.	.	2	.	4	0	1	4	5	.	.	0	.	5	5
54	4	1	3	.	3	4	2	2	5	3	4	1	2	4	5	1	.	3	1	5	> 5
71	.	2	.	1	.	.	.	1	.	.	.	0	.	.	.	3	.	1	.	.	.
75	0	.	.	.	.	3	3	2	4	.	4	.	2	4	.	.	.	3	.	5	.
77	0	.	.	.	.	4	.	2	.	.	4	4	.	4	2	.	.	4	.	.	.
80	2	.	5	.	.	.	5	.	.	5	.	.	.	.	.	.	.	2	.	.	.
2009	4	3	.	.	.	3	4	.	0	.	4	0	.	0	5	.	.	4	.	4	.
8719	3	2	.	.	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.
P.S.	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Dilutions of the phage giving minimal + + reactions on the test strains are compared with the dilution giving approximately the same reaction on the propagating strain. (P.S.) Recorded as follows:

5 = a ' + + ' reaction in the same dilution as on the propagating strain.

4 = a ' + + ' reaction in a dilution 10–10^2 times more concentrated than that giving a + + on the propagating strain.

3 = a ' + + ' reaction in a dilution 10^3–10^4 times more concentrated than that giving a + + on the propagating strain.

2 = a ' + + ' reaction in a dilution 10^5 times more concentrated than that giving a + + on the propagating strain.

1 = very weak lysis.

In general, a grade 3, 4 or 5 reaction where none existed previously or the complete absence of such a reaction where one should exist is an indication of a change in the phage preparation.

appeared in patterns with the old phages or with each other. A method of assessing redundancy of any of the phages was devised. One of two phages was considered redundant if the two phages always lysed the same culture of *Staph. aureus*. An ideal phage set is one in which there is as little redundancy as possible; when combinations of phage reactions occur, two phages must not invariably lyse the same culture of staphlococci. A further 2000 cultures of staphylococci were now typed by the basic set and the additional typing block of now 21 phages at RTD (after phage P3 had been added).

Table 6. *Numbers of cultures of staphylococci (out of* 2000) *lysed by the individual phages at different degrees of lysis*

Phage	+ +	+	Phage	+ +	+
29	361	30	190	397	44
52	476	44	191	127	94
52A	365	55	192	263	35
79	261	32	C 33	60	49
80	472	54	D 33	31	29
3A	38	1	Rosa	85	143
3C	59	3	SA 446	627	42
55	109	6	69	155	38
71	66	3	L 971	56	21
6	170	12	KS 6	106	20
42E	194	42	UC 13	117	52
47	229	18	86	194	64
53	225	37	E	34	17
54	225	25	C	264	62
75	307	32	75B	289	51
77	272	49	31	91	107
83A	171	28	44	121	53
84	355	41	52B	197	85
85	541	45	47A	2	4
81	419	30	75A	185	46
42D	22	9	P 3	200	29
187	4	0			

A modification of the sorting procedure designed by Davidson (1966) originally for computer analysis was now used. Patterns of lysis were coded and punched on standard 'Hollerith' punch cards on an International Computers and Tabulators (I.C.T.) Card Punch no. 129. The resultant holes were verified by a separate observer on an I.C.T. Verifier no. 129. Redundancy was detected by carrying out the following sorting procedure with an I.C.T. Sorter no. 302:

(1) Cultures were sorted into those lysed by each phage at RTD at two levels of lysis; + + (50 plaques or more) and + (20–49 plaques). The results at this stage are shown in Table 6.

(2) The cards sorted in (1) for each phage at the + + strength of reaction were then sorted for every other phage, again at the + + level of lysis.

(3) A similar sorting procedure to (2) was used but including all the + + and + reactions.

(4) A triangular matrix was constructed (Table 7) for the + + reactions, and a similar matrix (Table 8) for the combined + + and + reactions.

Table 7. *Matrix showing how many cultures of* Staph. aureus *were lysed by two phages at the same time at the + + level*

	29	52	52A	79	80	3A	3C	55	71	6	42E	47	53	54	75	77	83A	84	85	81	42D	187	190	191	192	C 33	D 33	Rosa	446	69	L 971	KS 6	UC 13	86	E	C	75B	31	44	52B	47A	75A	P 3
29																																											
52	247																																											
52A	133	319																																										
79	126	151	150																																									
80	187	369	318	131																																								
3A	1	5	6	4	7																																							
3C	1	5	6	4	6	21																																						
55	9	10	6	4	11	18	48																																					
71	6	0	0	4	0	12	33	46																																				
6	34	27	35	56	28	10	8	6	1																																			
42E	53	69	78	81	71	12	10	9	2	102																																		
47	43	30	38	53	31	13	8	8	5	146	113																																	
53	30	35	49	62	36	4	2	1	1	111	108	149																																
54	50	44	53	75	49	14	11	9	1	128	130	158	111																															
75	45	37	37	54	32	4	2	2	1	117	102	184	151	145																														
77	102	82	39	48	56	2	1	5	1	57	59	104	101	80	146																													
83A	23	36	47	50	38	11	9	8	1	71	80	83	92	79	70	46																												
84	15	17	24	30	17	1	0	0	0	30	37	69	82	48	119	122	58																											
85	52	44	52	63	46	11	9	9	1	90	81	130	128	116	182	125	99	308																										
81	96	195	173	91	248	13	11	13	8	100	135	108	77	112	92	67	65	21	58																									
42D	0	11	12	8	8	0	0	0	0	9	10	7	6	9	7	12	4	4	1	16																								
187	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0																							
190	232	261	162	116	216	12	11	14	1	28	67	35	24	46	40	86	32	14	55	145	6	0																						
191	39	61	74	70	64	11	10	10	1	26	47	29	15	42	28	16	37	13	36	65	3	0	80																				
192	92	109	100	66	140	5	4	5	1	59	81	77	45	97	81	42	30	23	65	165	7	0	105	35																			
C 33	25	36	40	33	41	7	6	6	2	23	34	26	19	29	20	10	19	6	14	52	4	0	38	38	28																	
D 33	7	13	13	16	14	3	3	3	0	14	17	18	17	23	15	11	7	6	18	20	0	0	12	15	16	16																
Rosa	36	33	26	17	32	0	0	5	6	14	20	26	25	18	24	28	12	15	34	24	6	0	48	14	17	10	5															
SA 446	252	377	264	135	393	11	9	18	7	41	67	59	31	63	68	110	32	19	65	293	10	0	291	78	161	42	12	35														
69	120	105	35	30	76	0	0	5	0	14	24	18	17	18	14	66	13	14	12	46	5	0	138	15	22	9	3	21	134													
L 971	12	9	12	9	7	1	1	0	0	42	33	49	20	50	48	27	36	16	35	33	0	0	7	2	28	1	1	4	14	3												
KS 6	27	41	34	10	71	0	0	0	0	17	12	22	4	23	21	20	3	4	15	83	0	0	22	7	59	2	1	3	92	8	7											
UC 13	9	11	20	19	11	1	0	0	0	40	46	75	99	45	77	63	55	56	74	29	3	0	2	1	13	5	1	15	7	6	24	1										
86	60	63	68	58	62	5	5	7	0	35	52	51	64	53	49	58	91	50	105	62	7	0	61	44	16	27	15	16	70	36	15	3	45									
E	6	14	0	12	17	7	7	7	0	13	17	12	8	20	6	1	8	0	22	18	3	0	10	12	13	12	4	2	16	1	0	1	0	8								
C	41	57	63	55	53	10	7	7	4	102	121	125	146	130	143	98	97	76	176	106	19	0	60	38	63	23	17	26	71	25	25	6	77	66	9							
75B	43	49	51	62	46	7	5	5	1	109	97	172	153	139	245	135	76	107	212	100	4	0	49	41	78	30	16	22	77	13	51	20	77	56	10	149						
31	88	80	61	50	75	1	1	4	0	8	28	12	14	18	10	32	11	11	15	38	3	0	72	28	22	20	4	20	74	39	3	2	3	42	6	20	12					
44	77	100	74	53	97	4	4	8	0	24	47	26	34	38	25	44	26	18	32	51	4	0	88	31	40	18	8	27	93	57	8	6	13	35	13	40	31	46				
52B	79	126	121	84	117	5	5	5	0	49	73	56	72	59	54	41	76	24	49	94	6	0	85	42	60	34	5	22	111	26	33	8	47	64	7	90	76	46	53			
47A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
75A	31	30	29	37	25	7	5	4	0	80	75	115	97	109	174	93	56	64	109	70	3	0	34	28	60	20	15	12	50	11	42	12	42	35	6	110	177	9	21	44	0	
P 3	12	15	23	24	18	0	0	3	2	61	48	86	105	49	85	66	90	114	146	37	0	0	5	10	20	11	1	15	13	2	36	2	73	63	1	76	91	1	16	50	0	52
	29	52	52A	79	80	3A	3C	55	71	6	42E	47	53	54	75	77	83A	84	85	81	42D	187	190	191	192	C 33	D 33	Rosa	446	69	L 971	KS 6	UC 13	86	E	C	75B	31	44	52B	47A	75A	P 3

Table 8. *Matrix showing how many cultures of* Staph. aureus *were lysed by two phages at the same time at both + + and + levels*

	29	52	52A	79	80	3A	3C	55	71	6	42E	47	53	54	75	77	83A	84	85	81	42D	187	190	191	192	C 33	D 33	Rosa	446	69	L 971	KS 6	UC 13	86	E	C	75B	31	44	52B	47A	75A	P 3
29																																											
52	268																																										
52A	169	352																																									
79	147	183	180																																								
80	230	426	350	156																																							
3A	1	9	8	4	7																																						
3C	1	9	7	4	6	23																																					
55	9	13	7	4	11	21	50																																				
71	6	1	0	4	0	12	33	47																																			
6	49	43	57	68	39	10	9	7	1																																		
42E	81	89	103	101	89	12	11	7	2	137																																	
47	60	45	61	65	48	14	9	10	5	156	142																																
53	58	61	79	87	65	6	6	3	1	124	140	167																															
54	78	66	84	92	69	15	12	11	1	146	162	181	140																														
75	67	54	61	69	49	5	4	3	1	124	124	192	170	161																													
77	141	109	64	63	92	2	1	5	1	62	86	116	122	99	166																												
83A	53	71	80	72	71	13	11	9	1	78	106	93	109	100	82	64																											
84	40	29	41	43	32	3	1	1	0	40	59	95	103	76	153	158	73																										
85	79	69	83	91	65	18	10	9	1	106	124	155	161	142	216	154	124	336																									
81	111	221	191	107	269	13	11	14	8	123	167	134	102	139	108	83	85	39	86																								
42D	5	17	18	9	12	0	0	0	0	14	17	13	14	16	15	19	12	11	6	22																							
187	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0																						
190	243	280	181	136	249	12	11	15	1	41	89	50	43	68	56	111	62	33	83	161	11	0																					
191	99	121	115	103	111	11	10	13	1	42	64	38	27	60	48	45	59	31	61	91	5	0	142																				
192	108	127	130	87	162	6	4	7	1	81	127	101	75	124	109	58	47	45	94	194	13	0	118	71																			
C 33	54	70	69	53	74	10	9	11	3	37	58	41	38	49	33	28	35	17	33	73	5	0	70	58	44																		
D 33	45	33	36	31	34	8	7	7	1	28	48	34	36	42	27	17	29	17	36	38	2	0	33	27	30	36																	
Rosa	116	110	65	49	106	0	0	7	6	52	62	72	80	65	69	112	42	50	80	75	15	0	127	43	48	31	13																
SA 446	273	417	297	159	435	11	10	19	8	47	84	70	55	84	97	146	57	42	88	309	13	0	322	139	185	75	29	113															
69	149	133	62	50	126	1	1	5	0	21	39	23	28	30	21	95	29	21	30	57	7	0	167	43	43	29	10	92	166														
L 971	28	21	25	19	22	2	1	1	0	58	50	68	32	68	66	44	45	29	51	47	4	0	16	14	51	10	7	27	30	11													
KS 6	31	51	45	16	85	0	0	0	0	21	21	27	10	29	24	23	7	11	21	97	0	0	29	14	68	9	4	13	105	11	20												
UC 13	23	24	32	35	27	2	1	0	0	65	80	105	148	77	113	102	80	87	110	59	10	0	14	9	30	17	11	57	21	15	40	6											
86	117	115	88	84	108	5	5	9	0	40	67	58	77	66	58	90	104	61	118	83	8	0	115	90	36	41	20	63	120	69	18	9	75										
E	7	27	18	21	28	9	9	9	1	22	26	20	13	29	12	2	11	1	26	31	6	0	17	22	22	20	14	10	27	3	0	2	3	10									
C	66	86	94	88	79	10	9	9	5	116	155	143	183	156	167	135	116	120	230	132	26	0	86	51	86	43	38	76	101	45	51	14	120	79	22	1							
75B	66	77	87	77	73	13	11	13	4	129	131	195	175	164	264	155	91	139	249	138	10	0	65	62	107	50	34	72	111	21	70	27	118	66	22	188							
31	184	163	95	82	149	1	1	6	1	16	48	19	34	33	18	77	34	19	24	64	4	0	150	74	42	47	15	95	157	111	9	13	13	95	7	42	22						
44	119	139	97	73	136	5	5	9	0	40	67	41	59	55	38	85	55	29	53	72	8	0	123	61	62	40	17	76	130	99	20	12	28	60	19	65	46	110					
52B	109	209	197	112	199	5	5	5	0	60	96	68	92	77	65	58	101	35	69	152	13	0	111	71	89	56	20	61	188	54	40	24	67	78	13	110	98	75	81				
47A	1	4	3	0	3	3	3	2	0	3	3	3	2	3	1	0	3	0	3	4	0	0	3	2	0	3	1	3	4	1	1	0	0	1	2	3	3	1	1	1			
75A	53	50	61	50	44	13	10	9	0	104	113	141	121	142	205	117	76	94	145	101	11	0	57	53	92	37	32	42	76	20	63	20	77	49	16	140	212	14	37	55	3		
P 3	27	27	36	40	33	0	3	2	3	70	71	102	127	56	96	74	99	124	172	58	4	0	21	24	35	20	9	67	23	12	40	8	100	78	2	103	108	14	32	59	0	60	

(5) A percentage matrix was then calculated from the results of the sorting procedure (2) as percentages of the numbers of cultures strongly lysed (+ +) by each phage (Table 6). The corresponding percentage matrix with the + + and + reactions combined was also calculated. Tables 9 and 10 show part of each of these matrices.

Table 9. *Part of the percentage matrix showing the results from Table* 7 *as a percentage of the total number of cultures lysed by each phage at the + + level.*

	29	52	52A	79	80	3A	3C	55	71	6
29	100	68	37	35	52	0	0	2	2	9
52	52	100	67	32	78	1	1	2	0	6
52A	36	87	100	41	87	2	2	2	0	10
79	48	58	58	100	50	2	2	2	2	21
80	40	78	68	28	100	1	2	2	0	6
3A	3	13	16	10	18	100	55	47	32	26
3C	2	8	10	7	10	36	100	81	56	14
55	8	9	6	4	10	17	44	100	42	6
71	9	0	0	6	0	18	50	65	100	1
6	20	16	20	33	16	6	5	4	1	100

Table 10. *Part of the percentage matrix showing the results from Table* 8 *as a percentage of the total number of cultures lysed by each phage at both the + + and + levels*

	29	52	52A	79	80	3A	3C	55	71	6
29	100	68	43	38	59	0	0	2	2	12
52	52	100	68	33	82	2	2	3	0	8
52A	40	84	100	43	83	2	2	2	0	14
79	50	63	62	100	53	1	1	1	1	23
80	44	81	67	30	100	1	1	2	0	7
3A	3	23	20	10	18	100	54	54	31	26
3C	2	15	11	6	10	37	100	81	52	15
55	8	11	6	3	10	18	43	100	41	6
71	9	1	0	5	0	17	48	68	100	1
6	27	24	31	37	21	5	5	4	1	100

The percentage matrices were now examined for each phage and any percentage of 50 or more was noted. Pairs of phages were identified which had 50–59, 60–69, 70–79 and 80 % or more similarities both ways. The lesser of the two percentages was taken as the 'both ways' answer. For example, the part of the + + percentage matrix shown in Table 9 shows that 67 % of the cultures of staphylococci lysed by phage 52 were also lysed by phage 52A, and 87 % of the cultures lysed by phage 52A were also lysed by phage 52. 67 % is therefore taken as the 'both ways' answer. Results were scored arbitrarily against each phage in the following manner: 80 % or more, 4; 70–79 %, 3; 60–69 %, 2; 50–59 %, 1. These scores were added separately for each phage both at the + + degree of lysis and for the combined + + and + degrees of lysis. The higher the score of a phage, the more redundant was that phage. The phage having the highest score was eliminated; in this experiment it was phage 47. The scores were reassessed in the absence of

phage 47 and the highest scoring phage was again eliminated, and so on. The next two phages to go were phages 52 and 75B.

A stage was now reached when phage 80 would have been eliminated. Consideration of the + reactions as well as the + + indicated that phages 53 and 54 would have been eliminated at the same time. This therefore appeared to be a useful place at which to draw the line. Ideally, phages should have been eliminated until the scores were all zero, but at this time the number of typable cultures would have fallen to an unacceptable level. Only two cultures became untypable following the rejection of phages 47, 52 and 75B.

Reproducibility

A comparison was made of the reproducibility of the phage-typing patterns obtained at RTD with the basic-set phages, the extra phages, and the two sets of phages used in combination. A series of pairs of cultures isolated within a short period of time from the same site were used for this purpose. A similar comparison of the patterns of lysis by the extra set of phages at RTD and the basic-set phages at RTD × 1000 was made in similarly related pairs of cultures that were untypable with the basic set of phages at RTD.

A survey of staphylococci carried by the patients and staff of St Bartholomew's Hospital included 782 pairs of cultures isolated from the nose swabs of the same person within a period of 5–10 days. About a third of these cultures were previously included in the 2000 cultures used in the assessment of redundancy. The additional cultures were retyped both with the basic set and with the extra phages. Cultures untypable at RTD by the basic-set phages were also retyped by these phages at RTD × 1000.

Williams & Rippon (1952) considered that a difference of two strong reactions between the phage-typing patterns of staphylococci, i.e. one culture lysed strongly (+ +) by two phages which gave no lysis of the other culture, or strong lysis of each of two cultures by a phage which gave no lysis of the other culture, was a good indication that the cultures came from two distinct populations of bacteria. These criteria were applied to the pairs of cultures above, and a comparison was made of the proportion of pairs which would have been considered 'the same' or 'different' when they were typed in different ways.

The numbers of pairs of cultures of staphylococci showing various differences in the pattern of lysis by the two sets of phages at RTD are shown in Tables 11 and 12. The differences are arranged into a series of categories (1–6) of increasing magnitude; pairs in which there was no common reaction (category 7) or in which one or both of the cultures were untypable (categories 8 and 9) are shown separately. In each of the tables, the first column gives the number of pairs of cultures showing differences in each category, and the second column gives the percentage distribution of these differences. In the third column, the percentages are recalculated when only strictly comparable cultures are considered, that is to say, after the exclusion of categories 7, 8 and 9, as suggested by Williams & Rippon (1952). It is reasonable to assume in most cases that when two cultures isolated from the same site within a short interval of time have no common phage

reactions, they are from different populations. When one or both of the cultures is untypable there is no way of assessing their relationship.

The percentages of presumably related pairs of cultures (column 3 in Tables 11 and 12) in which one culture is found to differ from the other by the two-strong differences rule were found for the two sets of phages at RTD. For the basic-set

Table 11. *Differences in the pattern of phage lysis of* 782 *pairs of cultures of* Staph. aureus *isolated from the nose swabs of the same person at an interval of* 5–10 *days*

(Phage-typing by the basic-set phages at RTD)

Category of the difference in phage pattern	(1) No. of pairs of cultures	(2) Percentage of 782	(3) Recalculated percentage (of 652)*
1. None	177	22·6	27·2
2. Strength of reaction only	73	9·3	11·2
3. Loss or gain of + or ± reactions	182	23·2	27·9
4. Loss or gain of one + +, with or without change in + and ± reactions	125	16·0	19·2
5. Loss or gain of two + +, with or without change in + and ± reactions	51	6·5	7·8
6. Loss or gain of three + +, with or without change in + and ± reactions	44	5·6	6·7
7. No common reactions	26	3·4	.
8. One culture untypable	59	7·6	.
9. Both cultures untypable	45	5·8	.

* Excluding categories 7, 8 and 9.

Table 12. *Differences in the pattern of phage lysis of* 782 *pairs of cultures of* Staph. aureus *isolated from the nose swabs of the same person at an interval of* 5–10 *days*

(Phage-typing by the extra phages at RTD.)

Category of the difference in phage pattern	(1) No. of pairs of cultures	(2) Percentage of 782	(3) Recalculated percentage (of 583)*
1. None	67	8·6	11·5
2. Strength of reaction only	71	9·1	12·2
3. Loss or gain of + or ± reactions	292	37·3	50·1
4. Loss or gain of one + +, with or without change in + and ± reactions	86	11·0	14·7
5. Loss or gain of two + +, with or without change in + and ± reactions	29	3·7	5·0
6. Loss or gain of three + +, with or without change in + and ± reactions	38	4·8	6·5
7. No common reactions	30	3·8	.
8. One culture untypable	53	6·8	.
9. Both cultures untypable	116	14·9	.

* Excluding categories 7, 8 and 9.

phages this percentage was 14·5 (7·8 + 6·7) and for the extra phage set it was 11·5 (5·0 + 6·5). If cultures of staphylococci with dissimilar antibiograms had been assumed to have come from different populations these percentages would

have been 13·6 and 11·3 respectively. Consideration of the close epidemiological relationship between the pairs of cultures leads us to believe that the extra phage set shows a greater stability of pattern than the basic set. However, it must be remembered that the basic-set phages type more cultures of *Staph. aureus* than those of the extra phage set, this having been the original reason for choosing the phages for the basic set.

Eighty-seven pairs of cultures of staphylococci which were untypable at RTD by the basic set and were therefore retyped at RTD × 1000 were now used to compare the reproducibility of the patterns produced at this strength of phage with those of the extra phages at RTD (Table 13). Of the comparable pairs of cultures (Williams & Rippon, 1952) 18 of 47 typed by the basic set at RTD × 1000 showed loss or gain of two strong reactions while no pairs of cultures of a similar group (0 of 55) typed by the extra phages showed these differences.

Table 13. *Numbers of pairs of cultures of* Staph. aureus *untypable with the basic set at RTD differing in their pattern of phage lysis when typed either with the extra phages at RTD or with the basic set at RTD* × 1000.

(Eighty-seven pairs of cultures isolated from nose swabs of the same person at an interval of 5–10 days were considered.)

Category of the difference in phage pattern	Extra phages typed	Basic set at RTD × 1000, ignoring reactions less than + −
1. None	17	12
2. Strength of reaction only	10	—
3. Loss or gain of + or ± reactions	20	—
4. Loss or gain of one + +, with or without change in + and ± reactions	8	17
5. Loss or gain of two + +, with or without change in + and ± reactions	0	18
6. Loss or gain of three + +, with or without change in + and ± reactions	0	0
7. No common reactions	6	3
8. One culture untypable	7	13
9. Both cultures untypable	19	24

Reproducibility of the individual degrees of phage lysis by the extra phages was not as good as that of the basic-set phages. The changes in the strength of reaction were mainly in the weak reactions which would rarely have changed the reported phage-type (Tables 11 and 12, category 3).

Discrimination

With the enlarged typing set, how many differences should be 'allowed' before cultures of staphylococci were considered to have come from different populations of bacteria? The percentage of pairs of cultures (see Tables 11 and 12) differing by two or more + + reactions when typed by the basic set and the extra phage set, respectively 14·5 and 11·5, equal approximately the percentage of pairs (11·9) differing by three or more + + reactions when typed by the combined sets

of phages (Table 14). It is therefore suggested tentatively that a 'three-strong differences' rule should be applied to patterns of lysis produced by the combined sets of phages much in the same way as the 'two-strong differences' rule suggested by Williams & Rippon (1952) is applied to the basic set alone at RTD.

Table 14. *Differences in the pattern of phage lysis of* 782 *pairs of cultures of* Staph. aureus *isolated from the nose swabs of the same person at an interval of* 5–10 *days*

(Phage-typing by the basic set and extra phage set combined at RTD.)

Category of the difference in phage pattern	(1) No. of pairs of cultures	(2) Percentage of 782	(3) Recalculated percentage (of 713)*
1. None	71	9·1	9·9
2. Strength of reaction only	43	5·5	6·0
3. Loss or gain of + or ± reactions	285	36·5	40·0
4. Loss or gain of one + +, with or without change in + and ± reactions	155	19·9	21·7
5. Loss or gain of two + +, with or without change in + and ± reactions	74	9·5	10·4
6. Loss or gain of three + +, with or without change in + and ± reactions	85	10·4	11·9
7. No common reactions	28	3·6	.
8. One culture untypable	29	3·8	.
9. Both cultures untypable	12	1·7	.

* Excluding categories 7, 8 and 9.

Table 15. *Number of distinct patterns of phage lysis among cultures* of* Staph. aureus *typed by three methods*

(*a*) with the basic set at RTD; '2-differences' rule applied. (*b*) with the combined set at RTD; '3-differences' rule applied. (c) with the basic set at RTD and RTD × 1000; '2-differences' rule applied.)

	No. of patterns	No. of cultures typable
Cultures typed by the basic set at RTD and the '2-differences' rule applied	136	183
Cultures typed by the combined set at RTD and the '3-differences rule' applied	150	192
Cultures typed by the basic set at RTD and RTD × 1000 and the '2-differences' rule applied	154	204

* Two hundred and eleven cultures were isolated in batches in one hospital on 10 occasions at an interval of one week; patterns for separate weeks were not compared.

There is a potential danger that the use of a 3-differences rule might reduce unacceptably the number of subdivisions which can be made between different cultures of staphylococci. The results of typing all staphylococci isolated from one ward of the St Bartholomew's Hospital survey in 10 consecutive weeks were then examined; these cultures formed part of the series of 2000 staphylococci previously typed. Numbers of distinct patterns of lysis observed in each separate week were added together (Table 15); patterns from separate weeks were not compared. On typing with the basic set alone at RTD, 136 patterns of phage

lysis were differentiable by the 2-differences rule; with the basic set at RTD and RTD × 1000, 154 patterns emerged. With the combined phage set at RTD, and the proposed 3-differences rule, 150 patterns were differentiable. In this example, 204 of 211 cultures were typable by the present system of phage-typing and 192 cultures were typable by the combined set of phages at RTD only. Thus, a roughly equal number of cultures were typable and differentiable with the two systems of typing and the different criteria for culture differentiation.

DISCUSSION

A system of phage-typing which relies in part on the use of phages at a thousand times their routine test dilution has four major disadvantages. First, inhibition reactions frequently occur and lead to confusion. Williams & Rippon (1952) found that some undiluted phage lysates caused inhibition of growth. When these lysates were progressively diluted, the effect was lost without passing through a dilution that produced plaques. Whether inhibition is a specific adsorption phenomenon or a non-specific lysis from without (Ralston, Baer, Lieberman & Krueger 1957) is still open to doubt. Burnet & Lush (1935) showed that coagulase-positive staphylococci adsorb all staphylococcal bacteriophages whether or not they were lysed by them. Rountree (1947) showed that coagulase-positive staphylococci adsorb all typing phages irrespective of their lytic activity and that this adsorption may be lethal. Ralston *et al.* (1957) described 'lysis from without' of *Staph. aureus* K_1 with phage-cell ratios greater than 17; this was independent of phage infectivity. It is probable that in staphylococcus phage-typing the ratio of phage to coccus in the area of the drop at RTD × 1000 is between 12:1 and 25:1.

The production of inhibition reactions depends on the relative concentration of phage and coccus (E. H. Asheshov, personal communication). Increasing the bacterial concentration may eliminate inhibition reactions and lowering it may increase the number and strength of these reactions. Beard & Rountree (1965) found that inhibition was due to phage/cell interaction when the phage/cell ratio was approximately 1:1. Most of the infected cocci were killed and a minority produced a few phage particles with a longer latent period. These phages are released and can only adhere to already dead cocci. There is therefore a generalized thinning of the bacterial growth. The evidence thus suggests (E. H. Asheshov, personal communication; Beard & Rountree, 1965) that inhibition reactions are very poorly reproducible and that very little epidemiological information is to be gained from them.

Secondly, at least 75% of the propagating strains are themselves lysogenic (Lowbury & Hood, 1953; Rosenblum & Dowell, 1960; Rountree, 1949) and concentrated lysates may be expected to contain not only the propagated phage but one or more temperate phages originally carried by the propagating strains.

Thirdly, phage preparations may also contain host-range mutants which may produce plaques on cultures insusceptible to the majority population of the phage. Dilution of the preparation to RTD will render the effect of these mutants negligible.

Fourthly, plating concentrated phage lysates on lysogenic bacteria may cause prophage induction with resultant plaque formation. In some cases this may be the cause of plaques appearing in an area of inhibited growth; these plaques must therefore be ignored. Blair & Williams (1961) suggested ignoring lesser degrees of lysis than 50 plaques and also all inhibition reactions, when typing at RTD × 1000.

In our experience, it is possible to type at least as many cultures of staphylococci untypable by the basic set at RTD by the use of 20 extra phages at RTD as were typed by the basic set at RTD × 1000, but these findings may not be applicable to cultures of *Staph. aureus* found in other parts of the world. Before any change in the internationally accepted procedure for phage-typing is undertaken a trial on a much bigger scale would have to be carried out.

With the growing doubts that the retyping of cultures of staphylococci at RTD × 1000 yields much additional information, it has been suggested that the use of a somewhat lower concentration of phage might improve reproducibility without reducing the percentage of typable cultures (Report, 1967). Jevons & Skalova at the Staphyloccocus Reference Laboratory (see Parker, 1966) studied the reproducibility of phage-typing at various concentrations of phage. They found that the reproducibility of phage-typing was much better at RTD × 100 than at RTD × 1000. An international study group has been formed to investigate typing results from all national laboratories at these two strengths of phage.

Degré (1966, 1967) has suggested that the use of the phages only at RTD × 100 is preferable to the present system. He found only slight degrees of inhibition at RTD × 100 and a greater reproducibility of the weak reactions. Zierdt & Marsh (1962), using this strength of phage, found that it 'tended to stabilize and complete phage patterns'. They also noted a marked absence of inhibition reactions. Whilst this stabilization of phage patterns might in some cases be advantageous, too much completion of phage patterns would tend to lump potentially unrelated cultures together and so reduce the epidemiological value of phage-typing.

The results of this investigation suggest that it might be preferable to substitute a number of extra phages at RTD for the secondary typing at RTD × 1000 of cultures untypable by the basic set at RTD. Cultures could either be typed by all the phages on the first day and differentiated by the suggested 3-strong differences rule, or the basic set and extra phages could be used on successive days and the 2-strong differences rule applied separately. The increased amount of spare room in the two typing blocks produces an incidental advantage in that there is more room for extra phages which may only have a local use for a restricted time. Future work on any expanded phage-typing system must be in the form of a comparative trial of a large number of phages in several countries. Adoption of any change in the method of phage-typing is a lengthy business and the proposed change, by using two blocks containing at least 40 phages, would necessarily require a world-wide trial.

SUMMARY

As many cultures of *Staph. aureus* untypable by the international basic-set phages at RTD were typed by a block of 20 extra phages at RTD as by the basic set at RTD × 1000. Reproducibility of the lytic patterns was as good with the extra phages at RTD as with the basic-set phages at RTD, and better than with the basic-set phages at RTD × 1000.

A method of assessing redundancy in any set of phages is presented, and a '3-strong differences' rule is suggested for discriminating between cultures of staphylococci from patterns of phage lysis by the combined set of phages at RTD.

Experience with the combined set of phages suggests that it may be possible to improve the phage-typing of staphylococci by substituting a larger series of phages used only at RTD, for the present set of 22 phages used at RTD and RTD × 1000.

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Investigation and treatment of floors of patients' rooms: a study with an agar cylinder

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INTRODUCTION

Nearly everywhere in patients' rooms bacteria can be isolated. Besides the air and textiles, the principal centres are smooth surfaces, such as floors, window-sills and similar places.

The flora found in these rooms often reflects not only that found on patients admitted, but also on personnel and visitors.

It is necessary to have at one's disposal a method which not only can ascertain contaminations accurately and at an early stage, but which can also determine the degree of contamination. The latter point is especially important in judging the effect of certain cleaning and disinfection methods.

Most investigators are satisfied, when testing, with a qualitative examination and use sterile cotton-wool containers moistened with broth (Noble & Lidwell, 1963). This method is very easy to apply, but gives little information on the number of bacteria found per unit of surface area. Examination of swept dust or dust from a vacuum cleaner is also easily carried out. However, this does not give quantitative data either. When sweeping, we do not collect the smallest particles, because these are distributed by this action through the air and keep floating there for a long time (Cruickshank, 1965). The smallest particles in particular, with a diameter of less than 2 μ, most often cause infection upon inhalation (Riley & O'Grady, 1961). The 'impinger method' used by Williams (1949) also only yields qualitative data.

Foster (1960) used 'impression plates' for the examination of floors. Gentles (1956) applied a replica technique. With the help of these methods both investigators were able to ascertain accurately the quantitative contamination of various parts of the floors of patients' rooms. However, both methods are rather complicated. Also, the 'agar sausage method' described by Ten Cate (1965) has its disadvantages, because the preparation of the sausages is difficult and time-consuming.

We have tried to find a method which is easy to apply, with which good results could be obtained qualitatively as well as quantitatively, and which could be used to compare the various cleaning and disinfecting methods and test their value. To this end we have devised an agar cylinder.

METHODS

Plate 1A shows the cylinder with accessories. The cylinder is closed with a lid. It, as well as the knife and sheath, is made of stainless steel and can easily be sterilized in an autoclave.

The agar cylinder is easy to handle. After sterilization the cylinder can be filled with various media, depending on the investigation we wish to carry out. Besides broth-agar we can use blood-agar or the medium of Chapman (1946) to isolate *Staphylococcus aureus*, glycerol-agar* for the isolation of *Pseudomonas aeruginosa*, while for the isolation of Gram-negative rods endo-agar can be used. In order to prevent the media from sliding out of the cylinder during the stamping, we have always used media prepared with 1·8 % Bacto-agar which were 1 day old. After the lid has been removed the medium is pushed 0·5 cm. out of the cylinder by turning the piston 2 or 3 strokes (Pl. 1 B). The medium is then pressed against the floor which is to be examined (Pl. 1 C). As the cylinder does not touch the floor, there is little chance of its being contaminated. With the knife a 0·5 cm. thick slice is cut off (Pl. 1 D) and placed in a Petri dish (Pl. 2 A). With one filled cyclinder about 20 tests can be done. The slices in the Petri dishes will be examined in the usual way after incubation.

RESULTS

An investigation has been carried out on the contamination of the floor with *Pseudomonas aeruginosa* in a patient's room at the respiratory centre in Groningen. This organism had been isolated at some time from the tracheobronchial aspirate of all four patients in this room. The examination of the floor with the cylinder filled with glycerol-agar showed that nearly all parts of the floor were contaminated with *Ps. aeruginosa* (Fig. 1). The contamination was especially high near the openings of the exhausts of the four Fricar suction pumps, which were at that time about 10 cm. from the floor. At the time of this investigation the floors were cleaned daily with soap and water. This treatment probably not only helped to spread the micro-organisms, but also gave them a chance to multiply. Thus we found fewer bacteria per surface unit immediately after treatment of the floor than 10 hr. later (Pl. 2 B, C). *Ps. aeruginosa* could not be isolated, after treatment of the floor with 0·3 % Halamid, for more than 12½ min.

To investigate the effect of daily sweeping only, a floor was sprayed with 1 ml./m.^{2} of a diluted broth culture containing 2×10^4 *Ps. aeruginosa* and $2{\cdot}3 \times 10^4$ *Staph. aureus* per ml. The floor was cleaned daily with a sterilized sweeper. Cylinders containing glycerol-agar and blood-agar were used for the isolation of the two organisms. The results of daily testing are shown in Table 1.

In Table 2 the results are given for a floor cleaned with water and a strongly alkaline soap (pH 9·5). This floor was sprayed with 1 ml./m.^{2} of a diluted broth

* The composition of the glycerol-agar used is as follows: 1 % (w/v) glycerol (= 0·81 % v/v), ½ % Difco proteosepepton, 0·04 % K_2HPO_4, 2 % $MgSO_4.7H_2O$, 0·001 % $FeSO_4$ (= 0·003% $FeSO_4.7H_2O$), pH 7·2–7·1. Add 1·8 % Bacto-agar and sterilize during 15 min. at a temperature of 120° C.

culture containing 2×10^4 *Ps. aeruginosa*, $2{\cdot}4 \times 10^4$ *Alcaligenes faecalis* and $2{\cdot}3 \times 10^4$ *Staph. aureus* per ml. Cylinders containing glycerol-agar, endo-agar and blood-agar were used for testing. The experiment was ended 150 min. after the floor was cleaned, at which time all three organisms could still be isolated.

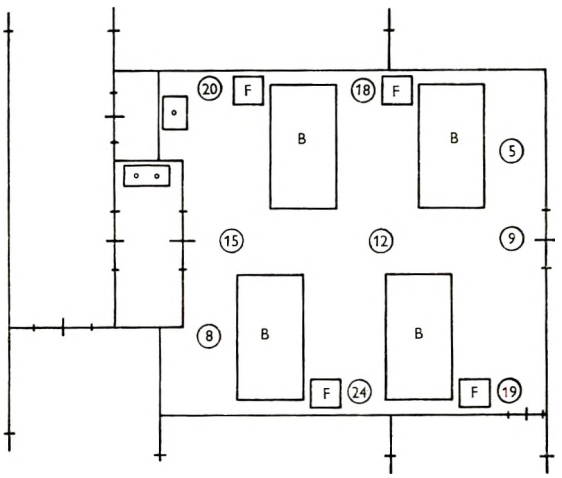

Fig. 1. The number of *Pseudomonas aeruginosa* colonies per print in various places in a room, into which four patients infected with *Ps. aeruginosa* had been admitted. B = bed, F = Fricar suction-pump.

Table 1. *Results of daily cultures from a floor that was swept daily for 17 days after infection*

Days after infection	Isolation of *Pseudomonas aeruginosa*	Isolation of *Staphylococcus aureus*	Days after infection	Isolation of *Pseudomonas aeruginosa*	Isolation of *Staphylococcus aureus*
1	+	+	10	+	+
2	+	+	11	+	+
3	+	+	12	+	+
4	+	+	13	+	+
5	+	+	14	+	−
6	+	+	15	+	−
7	+	+	16	−	−
8	+	+	17	−	−
9	+	+			

+ = growth; − = no growth.

In Tables 3–5 the results are given of experiments with floors cleaned with soap and water and then treated with one of three disinfectants, 0·3% Halamid (chloramine T), 2% liquor cresoli saponatus, or 2% Lyorthol. The infecting organisms and the method of testing were the same as those used in the previous experiment. To neutralize the disinfectants, 1% sodium thiosulphate was added to the medium in the experiments with Halamid, and 1% Tween 80 in the experiments with liquor cresoli saponatus or Lyorthol (Williams *et al.* 1966).

Table 2. *Results of cultures from a floor cleaned with water and a strongly alkaline soap, after infection*

Time after cleaning (min.)	Isolation of		
	Pseudomonas aeruginosa	*Alcaligenes faecalis*	*Staphylococcus aureus*
10	+	+	+
20	+	+	+
30	+	+	+
40	+	+	+
50	+	+	+
60	+	+	+
70	+	+	+
80	+	+	+
90	+	+	+
100	+	+	+
110	+	+	+
120	+	+	+
130	+	+	+
140	+	+	+
150	+	+	+

+ = growth.

Table 3. *Results of cultures from a floor cleaned with soap and water and disinfected with* 0·3 % *Halamid after infection*

Time after disinfection (min.)	Isolation of		
	Pseudomonas aeruginosa	*Alcaligenes faecalis*	*Staphylococcus aureus*
1	+	+	−
2½	+	−	−
5	+	−	−
7½	+	−	−
10	+	−	−
12½	−	−	−
15	−	−	−

+ = growth; − = no growth.

Table 4. *Results of cultures from a floor cleaned with soap and water and disinfected with* 2 % *liquor cresoli saponatus after infection*

Time after disinfection (min.)	Isolation of		
	Pseudomonas aeruginosa	*Alcaligenes faecalis*	*Staphylococcus aureus*
1	+	+	+
2½	+	+	+
5	+	−	+
7½	+	−	−
10	−	−	−
12½	−	−	−
15	−	−	−

+ = growth; − = no growth.

Table 5. *Results of cultures from a floor cleaned with soap and water and disinfected with 2 % Lyorthol after infection*

Time after disinfection (min.)	Isolation of		
	Pseudomonas aeruginosa	*Alcaligenes faecalis*	*Staphylococcus aureus*
1	+	+	+
2½	+	+	+
5	+	+	+
7½	+	+	+
10	+	+	+
12½	+	+	+
15	+	+	+
20	+	+	+
30	+	+	+
40	+	−	+
50	+	−	+
60	+	−	+
70	+	−	+
80	+	−	+
90	+	−	+
100	+	−	−
110	+	−	−
120	−	−	−

+ = growth; − = no growth.

DISCUSSION

With the agar cylinder method we were able to determine the degree of contamination in patients' rooms, and the influence of certain cleaning methods and the effect of various disinfectants could be accurately determined. It gives reliable information about the number of bacteria-carrying particles on floors. The number of bacteria in such a particle is usually small—about 4 per particle for *Staphylococcus* according to Lidwell, Noble & Dolphin (1959).

To what degree floors play a part in the starting of contaminations and infections cannot be definitely determined. Certainly infections form a serious problem in many hospitals. To counter them, it is necessary to lower the degree of contamination as far as possible. Because floors are to a certain extent gauges for the whole room, it is useful to test the floors. As floors have to be cleaned regularly, it is possible that the disturbance of the dust carries contamination from the floor. Because averting infections is meaningless unless all means of transport of infection are blocked, floors should be treated. In planning this treatment it must be remembered that damp floors increase the risk of *Pseudomonas* infections, since these organisms can multiply rapidly in a humid environment (Lowbury & Fox, 1953). On the other hand, dry floors often contain dust, which may not only be important as a secondary reservoir in infections with *Staph. aureus* (McDade & Hall, 1964), but in which *Ps. aeruginosa* can also live for a long time (Hurst & Sutter, 1966). When cleaning, both factors should be taken into account, which is not always easy. Some investigators advise the use of disinfectants besides

cleaning with soap and water, as the former cause a marked reduction in the number of bacteria (Ayliffe, Collins & Lowbury, 1966; Foster, 1960). We share their views. In our investigation Halamid and liquor cresoli saponatus gave satisfactory results, but the last of these cannot be used in patients' rooms because of its smell. We therefore prefer Halamid. Because this cannot be used on all floors, those floors should be favoured which are made of material not affected by Halamid, such as polyvynylchloride.

SUMMARY

A cylinder filled with medium is described, with which prints are made of floors or other surfaces. After a print has been made a slice is cut off the medium, put in a Petri dish and incubated. With one filled cylinder about twenty prints can be made. The method can be used for quantitative bacteriological testing of floors, and the effect of treatment with disinfectants can also be checked in a simple way. Preference was given to disinfection with Halamid.

I am obliged to Mr A. Feringa, who helped with the construction of the cylinder and with the examination of the floors, to Mr H. de Vries, who made the cylinder, and to Mrs H. F. v. d. Linden-Bonnema and Miss W. Kuiper for their assistance with the bacteriological investigation of the samples.

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J. S. KUIPERS

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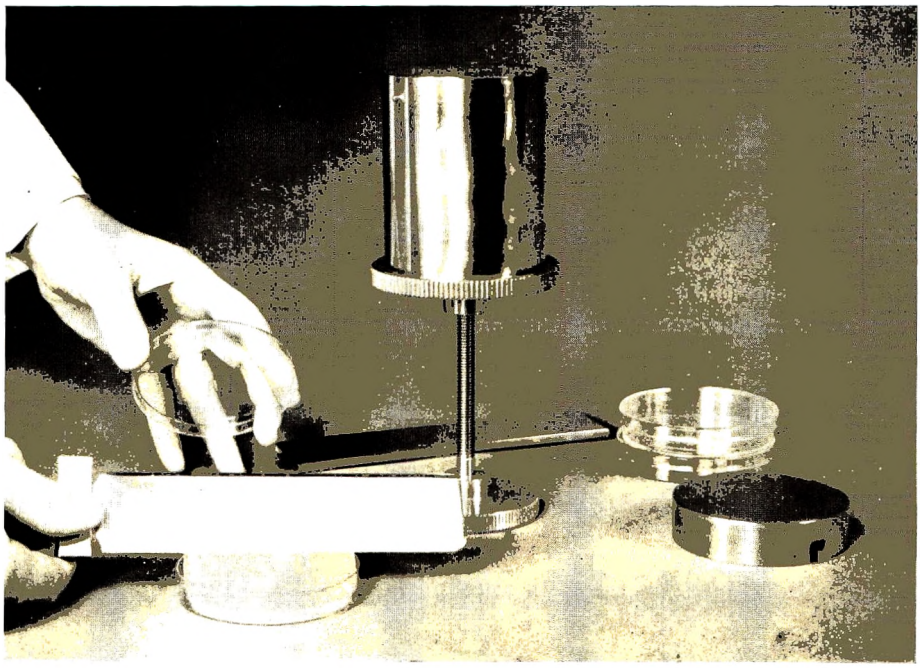

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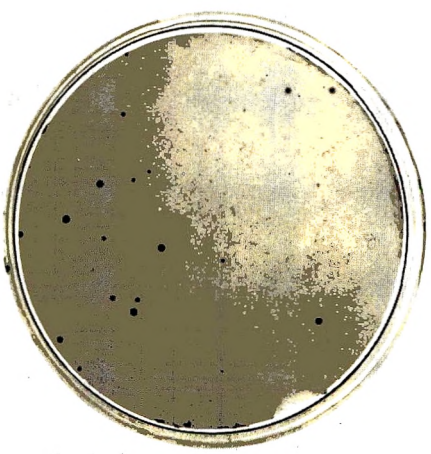

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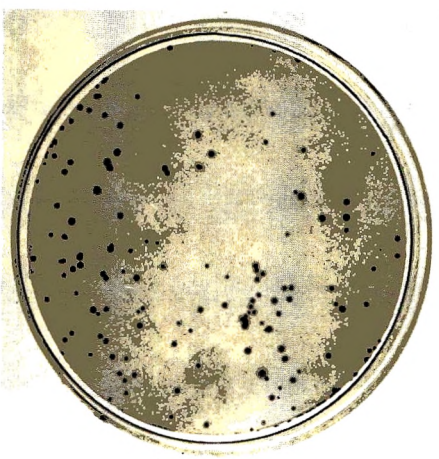

C

EXPLANATION OF PLATES

Plate 1

A Agar cylinder with accessories.

B Cylinder with medium pushed out 0·5 cm.

C The medium is pressed on the floor to be examined.

D A slice, 0·5 cm. thick, is cut off.

Plate 2

A The cut slice is placed in a Petri dish.

B Print of a floor cleaned with soap and water, immediately after treatment.

C Print of the same floor, 10 hr. after cleaning with soap and water.

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The persistence of foot-and-mouth disease virus in sheep

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van Bekkum *et al.* (1959), Sutmöller & Gaggero (1965) and Burrows (1966) showed that foot-and-mouth disease virus (FMDV) persisted in the majority of cattle for periods of several months after clinical disease. The possibility that FMDV also persisted in convalescent sheep and pigs was examined and this paper records the results of the experiments with sheep.

MATERIALS AND METHODS

Virus strains

(i) A–119	Pirbright stock cattle strain used at the 26th cattle passage
(ii) A–Iraq 24/64 (iii) O–Israel 1/63 (iv) SAT 1–S.A. 13/61	World Reference Laboratory samples received from the field and used at the 1st to the 3rd cattle passage.

Sheep

Commercial crossbred Southdown ewes, 8–15 months old, were used for the majority of experiments. The animals were housed in groups of four to eight in cattle boxes.

Infection was produced by either (i) the inoculation of the coronary band area of both main digits of one foot with $10^{4\cdot0}$ ID 50 (cattle tongue), or (ii) contact exposure to the inoculated sheep.

Examination for clinical lesions was carried out daily for 10 days. Three weeks after infection the animals were washed thoroughly and moved to a clean isolation unit.

Collection and handling of samples

Blood

Animals were bled daily for 4 days after inoculation, in-contact animals were bled on the 4th, 5th and 6th or 7th day. Further samples were taken periodically throughout the period of the experiment.

Oesophageal/pharyngeal samples

A modified version of the cattle sampling instrument referred to by van Bekkum *et al.* (1959) and described by Sutmöller & Gaggero (1965) was developed (Fig. 1). The volume of the sample obtained from the sheep varied between 0·1 and 0·3 ml. and this was collected from the instrument by rinsing in 2 ml. of diluent.

Post-mortem specimens

The regions sampled, the method of collection, and the preparation of specimens for infectivity assay have been described in connection with similar investigations in cattle (Burrows, 1966). The contents of the tonsillar sinuses and the tonsillar tissue were collected together by vigorous scraping from the pharyngeal surface. The rumen was examined and scrapings made from those areas which showed evidence of previous damage.

Isolation and infectivity assay of virus

All specimens were examined for infectivity within 1–3 hr. of collection. Blood samples were allowed to clot and only the serum was tested (previous work had established that the virus content of the serum did not differ significantly from that of whole blood during the viraemic stage).

All samples were assayed by counts of plaque-forming units (pfu) in baby hamster cell strain monolayers (BHK strain 21, clone 13, MacPherson & Stoker, 1962). Some samples were also examined for infectivity for primary monolayers prepared from calf thyroid (Snowdon, 1966). The identity of virus isolates was established 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).

RESULTS

Clinical observations

A detailed description of FMD following experimental infection of housed sheep has been given by Dellers & Hyde (1964), and of natural infection in pastured sheep by Zaikin (1959). Although classical lesions involving the mouth and feet may be seen following infection most workers have stressed that clinical manifestations of disease are frequently vague and difficult to demonstrate (Stockman & Minett, 1927; Viviano, 1957; Rivensen, Segura & Zakin, 1964; Cardassis *et al.* 1966; P. G. Howell & W. A. Geering, personal communications).

In the series of experiments recorded here only four of the 39 sheep developed obvious lesions in the mouth, and although the majority of animals displayed lesions on one or more feet, in general these lesions were mild and transitory in nature. A biphasic febrile response was recorded for the inoculated animals, but this was masked to some extent in those animals which acquired infection by contact.

Viraemia and antibody studies

The indeterminate nature of clinical lesions displayed by many infected animals has resulted in the use of other criteria to establish the presence of infection in sheep. The demonstration of circulating virus or a specific antibody response has been used for this purpose (Viviano, 1957; Dellers & Hyde, 1964; Rivensen *et al.* 1964; Cardassis *et al.* 1966; Fontaine, Dubouclard & Bornarel, 1966). Based on these criteria 38 out of 39 sheep, inoculated or exposed to virus, were shown to

have acquired infection. Details of the viraemia studies are presented in Table 1. Virus was recovered from the blood of the majority of the inoculated animals on each of the 3 days after infection, and from the in-contact animals on the 4th to the 7th day after initial exposure.

Neutralizing antibody appeared in the sera of the experimentally infected sheep 4 days after inoculation and reached peak levels after 7–10 days. Ten of the 11 sheep, exposed to virus by contact, acquired infection and developed significant levels of antibody within 11 days. The mean antibody levels of three of the four groups of sheep are recorded in Table 2.

Table 1. *Infectivity of blood (serum) after infection by or exposure to FMDV*

		Virus					
		A-119	A-Iraq		O-Israel		SAT 1
Method of exposure		CB*	CB	Contact	CB	Contact	CB
No. of animals		8	4	4	8	7	8
Days after exposure	1	8/2·90†	3/3·06	–	8/2·99	–	8/4·03
	2	6/4·31	4/2·70	–	8/3·59	–	8/5·04
	3	7/2·98	4/1·75	–	8/2·31	–	8/2·20
	4	2/0·85	0	3/2·00	0	4/1·80	0
	5	–	–	3/1·63	–	–	–
	6	–	–	0	–	5/2·35	–
	7	–	–	–	–	1/2·50	–

* Coronary band inoculation.
† Number of sheep with viraemia/mean infectivity of positive sera as $\log_{10}$ pfu/ml.
– Not tested.

Table 2. *Geometric mean neutralizing antibody titres of sheep used for 'carrier' studies*

		Virus				
		A-Iraq		O-Israel		SAT 1
No. of animals		4	8	8	14	8
Days after infection	0–3	0·8*	.	0·8	.	0·8
	4	–	.	1·7	.	1·7
	7	–	.	2·87	.	2·75
	11	2·4	.	2·90	.	2·79
	21	2·4	.	2·80	.	–
	28	.	2·47	.	2·63	3·08
	40–50	.	2·47	.	2·47	2·89
	70–90	.	2·33	.	–	3·00
	120–150	.	2·32	.	2·47	2·73

* Log_{10} reciprocal serum dilution (final).
– Not tested.

The infectivity of oesophageal/pharyngeal samples

Details of the frequency of recovery of virus and the mean titres measured in samples taken from the groups of sheep are shown in Table 3. Virus was recovered from approximately 80 % of animals 4 weeks after infection, from 45 % after 8

weeks, from 25% after 12 weeks and from one animal after 20 weeks. The mean infectivity of samples declined gradually from approximately 150 pfu/sample at 4 weeks (29 samples) to approximately 50 pfu/sample at 12 weeks (9 samples). The highest infectivity recorded for individual animals was greater than 1000 pfu/sample.

The results indicate that the duration of the carrier stage in sheep is much shorter than that observed in cattle infected with the same virus strains. The A-119 virus was recovered from five of 10 cattle for up to 7 months after infection and from one animal for more than 2 years. Two of four cattle infected with the A-Iraq strain yielded virus for a period of 14 months after infection.

Table 3. *The frequency of recovery of virus and the infectivity of pharyngeal samples from sheep after infection with FMDV*

		Virus			
		A-119	A-Iraq	O-Israel	SAT 1
No. of animals		7	8	14	8
Weeks after infection	2	7/1·71*	7/2·74	–	6/1·83
	3	7/2·48	8/2·54	8/2·16	–
	4	7/2·47	7/2·76	9/1·74	6/1·83
	6	6/1·87	7/2·1	8/2·20	5/1·86
	8	3/1·53	4/1·27	4/1·60	6/2·00
	12	1/2·2	4/1·90	1/1·40	3/1·50
	15	1/1·4	2/2·2	0	1/1·30
	20	0	1/0·3	0	0

* Number of sheep from which virus was recovered/mean infectivity of positive samples as $\log_{10}$ pfu per sample (BHK).
– = not tested.

Table 4. *Details of sheep examined* post mortem

Virus	No. of animals	History prior to infection	No. of weeks after killed	No. of animals from which virus recovered
A-119	1	None	3	1
	7		34	0
A-Iraq	8	Inactivated vaccine*	5–10	4
O-Israel	14	None	26	0
SAT 1	15	Inactivated vaccine†	3–6	4
	8	None	21	0
Total	53			9

* Challenged 21 days post-vaccination by coronary band inoculation.
† Challenged 21 days post-vaccination by tongue inoculation.

Sites of virus persistence

Information concerning the sites of virus persistence was obtained from two groups of vaccinated sheep which were killed several weeks after challenge with virulent virus. Three of the four groups of sheep which had been used for studies on the duration of the carrier stage were also sampled *post mortem* 5–8 months after

Table 5. *Virus content of specimens taken from sheep* post mortem

Virus	Animal identification	DPI*	Specimen: Soft palate	Specimen: Pharynx	Specimen: Tonsil
A-119	FK 76	21	0·6†	0·0	2·3
A-Iraq	FP 20	49	0·0	0·0	1·2
	FP 21		0·0	0·0	1·7
	FP 17	71	0·0	1·5	0·3
	FP 23		0·6	0·0	2·3
SAT 1	FM 6	30	2·7	2·5	3·0
	FL 95	36	0·0	0·0	2·0
	FL 97	36	0·0	0·6	0·3
	FL 98	37	2·6	1·2	4·0

* Days after infection.
† Log_{10} pfu/specimen (BHK).

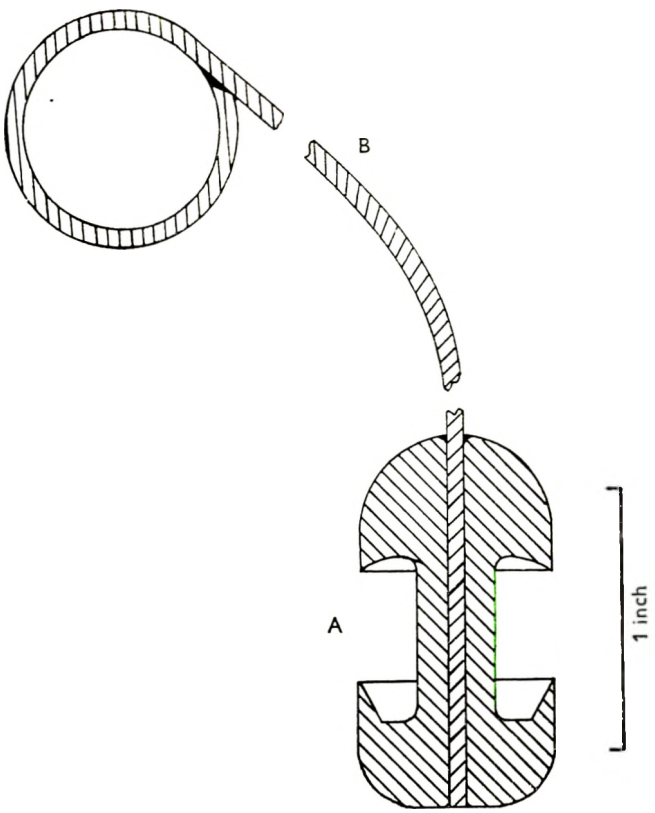

Fig. 1. Instrument for collecting pharyngeal samples from sheep. (A) Body machined from brass rod 0·75 in. O.D.; (B) stainless steel spring wire 16 SWG (0·064 in.), 12 in. long with finger loop.

infection, but no virus was recovered from these animals. Details of the numbers and history of the sheep used for this work are given in Table 4.

Virus was recovered from nine of the 53 animals examined. The distribution and concentration of virus found in these animals are given in Table 5. Virus was recovered most frequently and in highest titre from the tonsillar area and less frequently from the pharynx and the dorsal surface of the soft palate. No virus was found in samples taken from the nasal passages, the trachea or the rumen.

The distribution of virus in the carrier sheep differed slightly from that found in cattle in that the main concentration of virus in the sheep was in the tonsillar area, and in the cow the dorsal surface of the soft palate and the pharynx. This difference might be due to the fact that in some sheep the tonsils project into the *isthmus faucium* to a greater extent than they do in the bovine.

DISCUSSION

The clinical signs of disease in the sheep used in these experiments were mild and in many animals inapparent. This contrasts to some extent with the description of experimental infection in housed sheep reported by Dellers & Hyde (1964). However, Zaikin (1959) has observed that the frequency and character of lesions in sheep can be altered by environmental and climatic conditions. In our laboratory investigations the A-Iraq virus produced the most obvious signs of disease in sheep and the O-Israel virus the least obvious. However, the records of the World Reference Laboratory (J. Davie, personal communication) show that disease was reported frequently in sheep during the 1963 'O' outbreak but not during the 1964/1965 'A' epizootic.

In these and subsequent experiments no attempt was made to adapt virus to sheep. It was found that the inoculation of the coronary band area of susceptible sheep with cattle virus resulted in infection and the subsequent development of viraemia in all animals. This would appear to be a suitable challenge procedure for the evaluation of FMDV vaccines in sheep. Tongue inoculation of sheep with cattle virus (SAT 1–S.A. 13/61) did not initiate infection in all animals based on viraemia and serum antibody studies. This agrees with the findings of Fontaine *et al.* (1966), who demonstrated the necessity for passaging cattle virus in sheep in order to obtain regularity of infection by tongue inoculation.

The existence of a 'carrier state' in sheep following infection with FMDV was not unexpected in view of the findings for cattle and it would appear likely that FMDV persists in the majority of susceptible ruminants for variable periods of time after infection. The ability of the virus to persist in the pharyngeal area of the ruminant must signify a special virus/host relationship. No evidence for a 'carrier state' in either the pig or the guinea-pig has been obtained in a number of experiments, and repeated examinations of cattle following infection with strains of vesicular stomatitis virus (Federer, Burrows & Brooksby, 1967) showed no evidence of persistence of this virus in the pharyngeal area (unpublished work).

The difference in the sites of virus localization in cattle and sheep may be related to the virulence or attenuation of the virus strain for the particular species.

The original observations (Burrows, 1966) that cattle strains of virus could be recovered most regularly and in highest titre from the dorsal surface of the soft palate and from the pharyngeal walls have been confirmed in subsequent work with other cattle strains of virus. However, studies of the carrier state in cattle following vaccination with virus strains attenuated for cattle (to be published) have shown that in those animals in which virus persists localization of virus occurs mainly in the tonsillar region of the pharynx. It could well be that persistence of virus in the tonsillar region of sheep signifies that these strains are not particularly virulent for sheep. The clinical response of the animals to the viruses used in this study would support this hypothesis.

SUMMARY

Sheep infected with FMDV strains of different epizootiological origin developed a carrier state which persisted in the majority of animals for 1–5 months.

The sites of virus persistence and multiplication in the convalescent animal were identified by titration of suspensions of mucosae and epithelia taken *post mortem*. Virus was recovered most frequently and in highest titre from the tonsillar area and less frequently from the pharynx and dorsal surface of the soft palate. No virus was found in samples taken from the nasal passages, the trachea or the rumen.

I should like to thank Mr D. Goodridge for valuable technical assistance and Mr H. Smith, who made the collecting instrument.

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Confirmatory tests for coliform organisms

By the Public Health Laboratory Service Standing Committee on the Bacteriological Examination of Water Supplies*

(*Received* 16 *April* 1968)

INTRODUCTION

During a comparison of media for the isolation of coliform organisms from water (Report, 1968) it was necessary to know whether the reactions produced in the media were due to coliform organisms or not. One of the methods used for this purpose was the subculture of each tube showing a presumptive positive reaction to a tube of brilliant green lactose bile broth (BGB) which was then incubated for up to 48 hr. at 37° C. and examined for the production of gas (W.H.O. 1961, 1963). This was in addition to the subculture of each presumptive positive tube to BGB for incubation at 44° C. for the detection of *Escherichia coli*. Each presumptive positive tube was also subcultured to a MacConkey agar plate, so that if a tube of BGB did not produce any gas after 48 hr. at 37° C., colonies from the corresponding MacConkey agar plate could be picked to lactose peptone water (LPW) for incubation at 37° C. for up to 48 hr. A presumptive positive tube was regarded as giving a false reaction only if colonies picked from MacConkey agar failed to produce acid and gas from LPW after 48 hr. incubation.

It soon became apparent that a considerable number of presumptive positive tubes which on subculture failed to produce gas in BGB at 37° C. nevertheless produced colonies on MacConkey agar which were capable of producing acid and gas in LPW at 37° C. In view of this, BGB incubated at 44° C. for the detection of *Esch. coli* was investigated in the same way and similar discrepancies were discovered.

Since this work had all been done with a single batch of dehydrated BGB, similar experiments were carried out with other batches of dehydrated BGB and with BGB made up in individual laboratories. Although there were differences between these media, none of the BGB media detected coliform organisms from as many presumptive positive tubes as were detected by subculturing the tubes

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to MacConkey agar and picking individual colonies to LPW. Other liquid media were therefore investigated for possible use as rapid confirmatory media at 37 and 44° C.

MATERIALS AND METHODS

Choice of media for investigation

Since the majority of the organisms causing false presumptive positive reactions both in MacConkey broth and in glutamic acid media, particularly with samples of chlorinated water, are spore-bearing organisms (Report, 1968) the first medium tried was formate ricinoleate broth (A.P.H.A., 1960) which inhibits the growth of these organisms. It soon became apparent, however, that this medium was not satisfactory as a confirmatory medium since it allowed non-lactose fermenting organisms to develop and produce gas.

Some preliminary experiments in which formate was omitted from this medium gave promising results and it was therefore decided to investigate a lactose ricinoleate medium more thoroughly. In addition to a lactose ricinoleate medium containing 1 % lactose, it was decided to investigate a lactose ricinoleate medium containing 5 % lactose, since Lowe & Evans (1957) have shown that late lactose-fermenting organisms produce acid and gas more rapidly in a medium containing 5 % lactose than in one containing 1 % lactose.

Media used

The media used were: (1) MacConkey agar, (2) 1 % lactose peptone water, both prepared in accordance with the instructions given in Report no. 71 (Report, 1956), (3) Oxoid dehydrated brilliant green bile (2 %) broth (CM 31) prepared according to the manufacturer's instructions, (4) 1 % lactose ricinoleate broth and (5) 5 % lactose ricinoleate broth which were prepared as follows: peptone (Evans), 5 g.; sodium ricinoleate, 1 g.; lactose, 10 g. or 50 g.; distilled water to 1000 ml.; pH 7·6 sterilized by autoclaving at 115° C. for 20 min. after tubing together with a Durham tube.

Methods

From each tube giving a presumptive positive reaction two tubes of BGB, two tubes of 1 % lactose ricinoleate broth (1 % LR), two tubes of 5 % lactose ricinoleate broth (5 % LR) and a MacConkey agar plate were inoculated.

The MacConkey agar plate was incubated at 37° C. for 24 hr.

Tests at 37° C.

One tube of BGB, one of 1 % LR, and one of 5 % LR were incubated at 37° C. for 48 hr., and the results recorded. With all the media any amount of visible gas in the concavity of the Durham tube was regarded as positive.

If all the media gave a positive result no further tests were carried out. If all the media gave negative results or if there was any disagreement between them, one or more colonies were picked from the corresponding MacConkey agar plate

and inoculated into separate tubes of LPW which were incubated for 48 hr at 37° C. and examined for the production of acid and gas.

Tests at 44° C.

One tube of BGB, one of 1 % LR and one of 5 % LR were incubated at 44° C. in a water-bath for 24 hr. and the results recorded. Any amount of visible gas in the concavity of the Durham tube was regarded as positive. If all the media gave positive results no further tests were carried out. If all the media gave negative results or if there was any disagreement between them, one or more colonies were picked from the corresponding MacConkey agar plate and inoculated into separate tubes of LPW which were incubated for 24 hr. at 44° C. and examined for the production of acid and gas.

Table 1. *Comparison of brilliant green bile broth (BGB), 1 % lactose ricinoleate broth (1 % LR) and 5 % lactose ricinoleate broth (5 % LR) as confirmatory media for the coliform organisms*

	Tests at 37° C.				
	1 % LR + 5 % LR +	1 % LR + 5 % LR −	1 % LR − 5 % LR +	1 % LR − 5 % LR −	Total
BGB + LPW +	306*	2	1	—	309
BGB + LPW −	—	—	—	—	—
BGB − LPW +	12	—	1	—	13
BGB − LPW −	3	—	10	64	77
Total	321	2	12	64	399

* Since the other three media were in agreement these strains were not tested in LPW.

RESULTS

Tables 1 and 2 present the detailed results of 399 tubes giving presumptive positive reactions examined at one laboratory. Table 1 presents the results of tests at 37° C., Table 2 the results of tests at 44° C.

It can be seen from Tables 1 and 2 that there were no instances, at either temperature, in which a positive result was obtained in LPW when all three of the media under investigation gave negative results.

In the test at 37° C. for coliform organisms it can be seen from Table 1 that there were 29 instances in which the three media did not agree with each other. The major differences between the three media were the 12 true coliform cultures which gave positive results with 1 % and 5 % LR and negative results with BGB; and the 10 non-coliform cultures which gave negative results with BGB and 1 % LR but false positive results with 5 % LR. Thus 1 and 5 % LR are significantly better ($P \leqslant 0{\cdot}01$) than BGB in detecting true coliform organisms and of these two media 1 % LR is better than 5 % LR in showing up false positive reactions.

In the tests for *Esch. coli* at 44° C. it can be seen from Table 2 that there were 39 instances in which the three media under investigation did not agree with each other. The major differences were the 26 *Esch. coli* cultures which gave positive results with 1 and 5 % LR and negative results with BGB. Here again 1 and 5 % LR are significantly better ($P \leqslant 0{\cdot}01$) than BGB in detecting *Esch. coli* and of these two 1 % LR would appear to be better than 5 % LR in not giving false positive reactions.

Table 2. *Comparison of brilliant green bile broth (BGB), 1 % lactose ricinoleate broth (1 % LR) and 5 % lactose ricinoleate broth (5 % LR) as confirmatory media for* Escherichia coli

	Tests at 44° C.				
	1 % LR + 5 % LR +	1 % LR + 5 % LR −	1 % LR − 5 % LR +	1 % LR − 5 % LR −	Total
BGB + LPW +	168*	1	3	—	172
BGB + LPW −	—	—	—	2	2
BGB − LPW +	26	2	—	—	28
BGB − LPW −	1	—	4	192	197
Total	195	3	7	194	399

* Since the other three media were in agreement these strains were not examined in LPW.

Table 3. *Comparison of brilliant green bile broth (BGB), 1 % lactose ricinoleate broth (1 % LR) and 5 % lactose ricinoleate broth (5 % LR) as confirmatory media for coliform organisms and* Escherichia coli

	'Failures' in each of the three media			
Medium	False + ve	False − ve	Total false	No. of strains tested
	Tests at 37° C.			
BGB	0	13	13	399
1 % LR	3	2	5	399
5 % LR	13	2	15	399
	Tests at 44° C			
BGB	2	28	30	399
1 % LR	1	3	4	399
5 % LR	5	3	8	399

The number of failures in each medium at each temperature, assuming LPW to give the correct result, are shown in Table 3. Here again in the overall results 1 % LR is significantly better ($P \leqslant 0{\cdot}01$) than the other two media.

In view of the encouraging results with 1 % LR in this investigation, it was considered that much wider comparison of 1 % LR and BGB at both 37 and 44° C.

should be carried out. An investigation using the procedure given under Methods above, save that 5 % LR was omitted, was accordingly carried out in nine laboratories and the results in terms of 'failures' in each medium at each temperature are recorded in Table 4. There were considerable differences between the results obtained in different laboratories. The results, by laboratories, are set out in Table 5.

Table 4. *Comparison of brilliant green bile broth (BGB) and* 1 % *lactose ricinoleate broth* (1 % *LR) as confirmatory media for coliform organisms and* Escherichia coli

Tests carried out in nine laboratories. 'Failures' in each of the two media

Medium	False +ve	False −ve	Total false	No. of strains tested
		Tests at 37° C.		
BGB	12	139	151	2447
1 % LR	42	91	133	2447
		Tests at 44° C.		
BGB	8	151	159	2822
1 % LR	16	50	66	2822

DISCUSSION

In one laboratory comparisons at 37° C. were made at 24 hr. It was apparent, however, that 24 hr. at 37° C. was not a sufficiently long period of incubation even for 1 % LR and this point was not pursued further.

In the results recorded in Table 4, 1 % LR is significantly better ($P \leqslant 0{\cdot}01$) than BGB at both 37 and 44° C. and where 1 % LR fails it is in calling a number of false presumptive positive reactions true coliform reactions. This at least is an error in the right direction since in water bacteriology the most important point is not to miss any true coliform reactions.

It will be seen from Table 5 that in the majority of the laboratories taking part in the investigation 1 % LR was better than BGB. It should be borne in mind also that the number of presumptive positive tubes in which either medium gave a false result was small. BGB gave the correct answer in 2296 (93·8 %) tubes out of 2447 at 37° C. and 2663 (94·4 %) out of 2822 at 44 °C, whereas 1 % LR gave the correct answer in 2314 (94·6 %) out of 2447 tubes at 37° C. and 2756 (97·7 %) out of 2822 at 44° C.

It is apparent, therefore, that in general either BGB or 1 % LR would be satisfactory as a confirmatory medium at both 37 and 44° C. It may be that the different results obtained in the different laboratories taking part in the investigation were largely due to the nature of the organisms in the samples examined. In certain samples the difference between the results in the two media was quite striking, and it may be that the choice of which medium to use is one that will have to be made by each laboratory depending on the known behaviour of the waters that it is examining. It is, however, true that over-all 1 % LR produces fewer false negative reactions than BGB at both 37 and 44° C and that its use as an alternative confirmatory medium to brilliant green bile broth can be recommended.

Table 5. *Confirmatory tests*

Comparison of brilliant green bile broth and 1% lactose-ricinoleate broth at 37 and 44° C.
Results in individual laboratories

Laboratory	False reactions at 37° C.			Medium	False reactions at 44° C.			No. of strains examined	
	Total	False +	False −		False −	False +	Total	37° C.	44° C.
Oxford	39	1	38	BGB	62	2	64	825	834
	9	5	4	1% lactose ricinoleate	7	1	8		
Newport	9	0	9	BGB	7	0	7	119	566
	7	0	7	LR	7	0	7		
Southend	8	5	3	BGB	—	—	—	175	0
	28	17	11	LR	—	—	—		
Brighton	24	0	24	BGB	2	0	2	334	334
	23	5	18	LR	0	1	1		
Hull	4	0	4	BGB	1	0	1	139	139
	3	1	2	LR	0	1	1		
Conway	29	5	24	BGB	26	5	31	346	348
	36	10	26	LR	24	10	34		
Metropolitan Water Board	2	1	1	BGB	0	0	0	66	158
	2	1	1	LR	0	1	1		
Wakefield	3	0	3	BGB	9	0	9	38	38
	1	0	1	LR	3	0	3		
Manchester	33	0	33	BGB	44	1	45	405	405
	24	3	21	LR	9	2	11		
Totals as already analyzed	151	12	139	BGB	151	8	159	2447	2822
	133	42	91	LR	50	16	66		

SUMMARY

In nine laboratories 1 % lactose ricinoleate broth has been investigated as a possible alternative to brilliant green bile broth as a confirmatory medium for coliform organisms and *Escherichia coli*.

Although the results varied considerably from one laboratory to another, in the sum of the results and in the majority of the participating laboratories 1 % lactose ricinoleate produced fewer false negative reactions than brilliant green bile broth as a confirmatory medium at both 37 and 44° C.

Although both media are satisfactory, 1 % lactose ricinoleate broth can be recommended as an alternative to brilliant green bile broth for the confirmation of coliform organisms and *Esch. coli* from presumptive positive tubes in the examination of water samples, as it is not subject to the known variability of brilliant green and ox-bile.

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An outbreak of Q fever in Staffordshire

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INTRODUCTION

Q fever was first recognised in 1935 in Brisbane (Australia) and was referred to as 'abattoir fever' (Derrick, 1937). In 1937 Burnet & Freeman showed that the infection was due to *Rickettsia*. During World War II the disease caused widespread sickness among members of the allied forces operating in the Mediterranean area and subsequently the disease has been found to be world-wide in distribution.

Incidence of confirmed cases occurring in England and Wales has been reported by the Public Health Laboratory Service as follows (Vernon, 1967):

1962 (53), 1963 (31), 1964 (30), 1965 (33), 1966 (32).

In this country it has been recognized for some time that the disease is associated mainly with sheep and cattle. Sheep farmers were shown to be at special risk in the lambing season when the infection is acquired from the infected placenta (Marmion & Stoker, 1956). Dairy farm workers are likewise at risk during calving, but this has no seasonal incidence, as calving takes place all the year round. Dust from the byre has been shown to be the source of infection on several occasions and infected dust from straw was held to be responsible for an outbreak in East Kent among the students and staff of a college (Harvey, Forbes & Marmion, 1951).

Case-to-case infection is uncommon but has been described in a small outbreak among R.A.F. personnel when four attendants in the sick bay and one visitor to the sick bay were all infected from a patient who had acquired his original infection on a farm in Cornwall (Holland *et al.* 1960).

In another outbreak involving R.A.F. personnel, the men affected had helped to clean out sheds into which sheep had strayed and probably lambed. The infection was presumed to have been acquired from the infected dust (Holland *et al.* 1960).

Raw milk has been suspected as the vehicle of infection on more than one occasion. Marmion & Stoker (1950) stated that those more likely to be affected from this source were town dwellers who drink non-pastuerized milk on a visit to the country. Marmion & Harvey (1956) reported 23 sporadic cases in Kent between 1948 and 1954 which they concluded had probably been infected by milk.

Evans (1956) recorded that approximately 5 % of milk samples from individual herds in South Wales were infected with *R. burneti*.

Huebner & Bell (1951) recorded that 10 % of dairy cows in Los Angeles County area were infected with *R. burneti* and that butter made from unpasteurized milk, from a known infected herd, was itself infected.

Unpublished work of one of us (W. L. H.) has shown 18 % of herds from which raw milk is retailed within the county of Staffordshire to be infected with *R. burneti*.

THE OUTBREAK

The Detention Centre in which the outbreak occurred is situated 4½ miles from the nearest town centre near open farm land and is about 700 ft. above sea level. The main building is of stone with outhouses, workshops and farm buildings. The standard of hygiene in the Centre and on the farm is of a very high order and rodents are rarely seen. The Centre has accommodation for 100 boys and in March and April 1967 the daily population varied from 95 to 99. The average length of stay is 10 weeks and the age range of detainees is from 18 to 21 years. There is a staff of twenty officers and some clerical assistants. All entrants are given a number on the day of admission, provided with clean clothing and bedding and then medically examined. Their first night is spent in separate cubicles and then they are transferred to the dormitories. The new entrants are purposely distributed throughout the establishment for administrative reasons.

Detainees are employed in the Centre in one of three ways: (i) domestic work within the Centre, (ii) agricultural work in the fields or tasks connected with animal husbandry, (iii) in manufacturing concrete blocks, fence supports, etc.

The outbreak was explosive in nature and occurred between 15 and 29 April 1967.

Boys began reporting sick from 15 April onwards and the clinical picture they presented was remarkably uniform. In practically every case there was high temperature ranging from 101 to 105° F associated with shivering, sweating and backache. Sore throat, headache, chest pains and neck stiffness were also common. Most had an irritating cough. X-ray examination revealed consolidation of the lungs in five cases. Three cases had transient, diffuse rashes on the trunk, of a fine punctate erythematous nature.

The duration of pyrexia varied from 2 to 7 days. No organized trial of antibiotic therapy was carried out but approximately half the patients were treated with tetracycline and the remainder had none. The general impression was that both groups progressed equally well. All patients were isolated, kept in bed and treated with aspirin only, except for those who also had tetracycline. Recovery was quick in most instances owing probably to the patients being fit young men with good physique.

One patient who was definitely more ill than the others was admitted to the local isolation hospital. He had a temperature of 105° F. on admission. He was shivering and this was followed by profuse sweating. He complained of headache, backache and pain in the loins. He had a slight cough but there were no obvious physical

signs in the chest. Crepitations became audible in the right lung 48 hr. after admission. The liver was not enlarged but the spleen was palpable. There was no enlargement of lymph glands and no rash was present. The blood picture was uninformative except for a raised E.S.R. X-ray of the chest showed pneumonic consolidation at the right base. He was treated with tetracycline and the temperature fell to normal within 72 hr. It was 6 weeks before the lungs were radiologically clear. A total of 24 boys and five officers suffered a clinical illness but a full investigation was not possible in all of them. Some of the boys were discharged before second specimens of blood for serology had been obtained. Follow up of these patients proved difficult. It was, however, possible to show serological as well as clinical evidence of Q fever in nineteen boys and five officers.

LABORATORY INVESTIGATION

The advice of the virologist from the local Public Health Laboratory was sought as soon as it became clear that an epidemic of unknown cause was occurring.

Blood specimens and throat swabs were taken from nine of the inmates who were ill on 21 April 1967. The sera were separated and stored at $-20°$ C. The swabs were broken off in transport media containing Hanks's basal salts solution, 0·2 % bovine albumen and antibiotics at the bedside and transported immediately to the laboratory in a flask of melting ice. On receipt, equal volumes of the transport media were inoculated into tissue culture tubes containing monolayers of monkey kidney, human embryo kidney, HeLa and W.I. 38 fibroblast cells. Tubes were incubated at 33° C. and examined over a period of 21–28 days for cytopathic changes and also for haemadsorbing properties using guinea-pig blood cells at 4° C. and at room temperature. No viral agents were detected.

Table 1. *Tests on paired serum specimens from patients*

Case no.	Register no. of inmate	Q fever C.F. antibody titre 21.iv.67	3.v.67	Biological test results Guinea-pig serology
1	637	< 1/5	1/80	Negative
2	647	< 1/5	1/160	Positive for *R. burneti*
3	672	< 1/5	1/80	Negative
4	673	< 1/5	1/160	Negative
5	674	< 1/5	1/320	Positive for *R. burneti*
6	676	< 1/5	1/80	Negative
7	628	< 1/5	< 1/5	Negative
8	677	< 1/5	1/160	Negative

When it seemed that it was unlikely that an agent would be isolated it was decided that an attempt should be made to diagnose the disease by serological methods. Convalescent sera were obtained from seven of the nine patients bled on the earlier occasion. The remaining two had completed their stay at the Detention Centre and had been released. Acute and convalescent sera from the patient admitted to the isolation hospital was also available. Complement-fixation tests were carried out in 80 hole plastic agglutination trays by the method of Bradstreet

& Taylor (1962) using 3 M.H.D. complement, and antigens supplied by the Standards Laboratory. Antigens employed were Influenza A, B and C, Sendai, Psittacosis/L.G.V., Adenovirus, respiratory synctial virus, Q fever (*R. burneti*) and *Mycoplasma pneumoniae*. None of the pairs of sera showed rises in antibody titre except to Q fever. Fourfold or greater rises in complement-fixing antibody were shown in seven of the eight patients as shown in Table 1.

When these results were known it was decided to inoculate guinea-pigs with 1 ml. amounts of the acute phase sera which had been stored for 2 weeks at $-20°$ C. The specimens were inoculated intramuscularly into the thigh and the animals were bled and killed after a period of 4 weeks. Presence of a high level of complement-fixing antibody in the guinea-pig serum was taken as an indication that the inoculum had contained live *R. burneti* organisms. This was demonstrated in two patients (Table 1).

Convalescent sera from 11 out of 14 other inmates who were clinically ill at the same time as those which were fully investigated showed complement-fixing antibody at a titre 1/20 or more. At the same time eight out of nine sera from inmates who had had no symptoms had antibody titres of less than 1/5. Only one symptomless inmate had a raised antibody titre of 1/60 and he denied having experienced anything which could have been attributed to infection.

When it was established that the epidemic was due to Q fever, the source of infection was looked for and the obvious possibility was the farm and milk produced from the dairy herd.

The possibility of environmental contact with infected animals was fully investigated but it was found that, although the centre had a herd of cows and a number of pigs, none of the sick inmates had been engaged in duties which brought them in contact with any of the animals in the 3 weeks before the outbreak. The one outstanding feature was that milk from the farm was consumed raw on one or two mornings weekly when cornflakes were served at breakfast. All other milk used in the kitchens was subjected to some form of heat during the cooking process and cold milk drinks were never issued. Officers at the Centre did not drink the raw milk or eat meals prepared in the kitchen but there was evidence that they had drunk cups of tea containing the untreated milk and it must be assumed that the organism was able to survive the temperature of the hot tea. Officers' families purchased heat-treated milk from local retailers and excess milk from the farm at the Centre was always sold to a large dairy, where it was duly pasteurized. One officer, who was clinically and serologically a definite case, denied ever consuming milk in any form. Huebner *et al.* (1949) showed that naturally infected milk failed to yield *R. burneti* after high-temperature–short-time heat treatment at $160{\cdot}5°$ F. $\pm$ $0{\cdot}3°$ F. for 15 sec. but that the organism could survive 35 min. at a temperature of $143°$ F $\pm$ $0{\cdot}5°$ F.

The farmer and his wife and the two regular estate hands never drank the raw milk but did have constant contact with the farm animals. None of them became infected clinically or serologically with the disease.

Milk being the probable source of infection, it was arranged with the veterinary surgeon to have blood samples from each milking cow examined for Q fever

antibody and milk from each cow injected into guinea pigs. The results in Table 2 show that, of the 20 cows examined, two were actively excreting *R. burneti* in their milk and three others had Q fever antibody titres of 1/10 or more in their serum. The opportunity was also taken to examine the milk for the presence of *Brucella abortus*. These results are also shown in the table. Although brucella organisms were not isolated from any of the milk specimens, the Ring Test for brucella antibody was positive in the milk from five animals.

Table 2. *Investigations carried out on milking cows*

Cow no.	Cow serum Q fever antibody titre	Tests on guinea-pigs after injection of milk			Direct milk tests		Months since parturition
		Q fever CFT	*Br. abortus* agglut.	*Br. abortus* culture	Brucella ring test	Brucella culture	
1	–	–	–	–	–	–	3
2	–	–	–	–	–	–	0
3	1/10	–	–	–	–	–	2
4	–	–	–	–	+	–	0
5	–	–	–	–	+	–	3
6	–	–	–	–	–	–	2
7	–	–	–	–	–	–	8
8	–	NS	NS	NS	NS	NS	·
9	–	–	–	–	–	–	2
10	–	+	–	–	–	–	3
11	–	–	–	–	+ +	–	1
12	1/10	–	–	–	–	–	10
13	–	–	–	–	+	–	9
14	–	–	–	–	–	–	11
15	–	–	–	–	–	–	9
16	–	–	–	–	–	–	3
17	1/10	–	–	–	–	–	9
18	1/20	+	–	–	+ +	–	13
19	–	NS	NS	NS	NS	NS	·
20	–	NS	NS	NS	NS	NS	·

NS = no specimen of milk available.

As an emergency measure, early in the outbreak, arrangements were made for all milk to be boiled. When it was established that the milk from the herd contained live Rickettsia organisms, the authorities arranged that all the milk produced on the farm should be sold for pasteurization and a heat-treated supply obtained for the needs of the establishment.

CONCLUSIONS AND OBSERVATIONS

This outbreak is of particular interest because it occurred in an institution where the activities of the inmates were under strict control.

The explosive nature of the outbreak and the scatter of the cases throughout the institution was very much against the infection being from a human source.

The fact that most of those affected were known to have had no direct contact with the dairy farm excluded the possibility of a dust-borne infection.

Spread by parasitic insects could be completely excluded, as there was no infestation among the inmates. A high standard of hygiene is maintained in the institution and enforced personal cleanliness of the inmates makes such an occurrence very unlikely.

Food-borne infection appeared to be the only likely explanation of the outbreak. The only food substance possible in this particular case was raw milk and this prompted us to investigate the cattle in the institution farm which supplied the raw milk. The evidence obtained from this investigation points strongly to milk as the vehicle of infection and we believe this to be the first milk-borne outbreak of Q fever to be reported in Britain.

Clinically the outstanding features were the short duration of symptoms even when no antibiotic was given, and the low incidence of pulmonary complications.

Q fever is yet another of the health hazards associated with raw milk. Heat treatment of all milk supplies is the only health policy compatible with safety.

It is a curious anomaly in this country that retailers are allowed to charge more for raw milk than for pasteurized or sterilized milk. The consumer may indeed be paying for something extra but it is often not quite what he expects.

All milk in Britain to-day comes from Tuberculin Tested herds, but there is no guarantee that untreated milk is free from typhoid and other salmonella organims, brucella, Q fever, streptococci, staphylococci or faecal bacteria. Most milk sold in urban areas is heat-treated but raw milk is still widely available in rural and semi-urban districts. Q fever must clearly be considered when outbreaks of obscure pyrexia occur in such areas.

Evans, Powell & Burrell (1959) reported a fatal case of endocarditis associated with Q fever. Recent published work of Grist, Ross & Sommerville (1967) and Kristinsson & Bentall (1967) has implicated *R. burneti* as a cause of subacute endocarditis. If a patient is, therefore, known to have had Q fever and later in life develops an unexplained low grade febrile illness, the possibility of rickettsial endocarditis should be borne in mind.

SUMMARY

This article describes an outbreak of Q fever involving 24 men (five prison officers and nineteen detainees) in one of H.M. Detention Centres. The evidence collected points strongly to the consumption of raw milk as the route of infection. This appears to be the first milk-borne outbreak of Q fever to be reported in Britain.

We wish to express our thanks to the Director of the Prison Medical Service, Home Office, for permission to publish; to Mr R. B. Wood, M.R.C.V.S. for his help in the collection of milk and blood samples from the cows and to Mr C. F. Kershaw, M.R.C.V.S., Ministry of Agriculture and Fisheries Veterinary Investigation Officer, Tettenhall, for assistance with the laboratory investigations.

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Acute glomerulonephritis in Trinidad: serological typing of group A streptococci

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INTRODUCTION

The recent epidemic of acute glomerulonephritis in Trinidad is the third to be recorded there in the past 20 years (Symonds, 1960, Poon-King *et al.* 1967). The number of persons who suffered from the disease in the years 1964–6 probably exceeded 2000; between September 1964 and April 1966, 720 cases of acute nephritis were admitted to San Fernando General Hospital (Poon-King *et al.* 1967) and a further 528 to the Paediatric Department alone of Port of Spain General Hospital.

The first evidence that epidemic nephritis in Trinidad was a poststreptococcal disease, and that the primary streptococcal lesion was more often of the skin than of the upper respiratory tract, was obtained in San Fernando (Simon *et al.* 1965). In March 1965, 21 hospital patients with acute nephritis were examined; 18 of them had skin sepsis, and group A streptococci were isolated from the skin, nose or throat of 16 patients. Potter, Moran, Poon-King & Earle, (1967) have since examined the serological characters of 25 strains of group A streptococci isolated at that time, and have described a new M-type of group A streptococcus (provisional type 55) thought to have nephritogenic properties.

In September 1965, one of us (D. C. J. B.) arrived in Trinidad as a member of the staff of the Trinidad Regional Virus Laboratory, University of the West Indies, and began a study of the bacteriology of nephritis and skin sepsis. His work in Trinidad was carried out at first in the Trinidad Regional Virus Laboratory and later in the laboratories of the Port of Spain General Hospital.

We now report observations on the typing of the group A streptococci that were isolated from cases of nephritis, and of skin sepsis uncomplicated by nephritis, in the first year of this study and during the previous 6 months. The part played by streptococci of type 49 (the Red Lake streptococcus) as a cause of nephritis in Trinidad has already been described briefly (Maxted, Fraser & Parker, 1967).

MATERIALS

The population of Trinidad is concentrated chiefly in the western part of the island (see Fig. 1). Port of Spain and San Fernando have large general hospitals, which constitute the main part of the hospital service for the whole island. Each

can be regarded as serving about 400,000 persons. Nearly all the patients with nephritis on whom we made bacteriological observations had been admitted to one of these hospitals, but a few were in the small district hospital at Arima, about 20 miles east of Port of Spain.

Acute glomerulonephritis

We report the results of typing cultures of group A streptococci from a total of 117 cases of acute glomerulonephritis.

From the Port of Spain and Arima hospitals, in the period September 1965 to August 1966, we collected 133 cases in which swabs were obtained, and for whom the presence of oedema, albuminuria and haematuria was recorded; when the

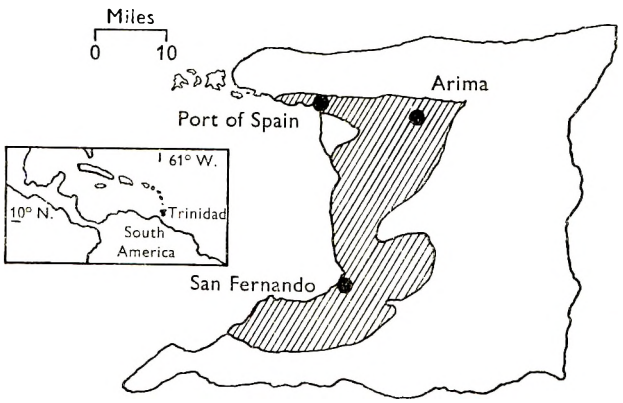

Fig. 1. Map of Trinidad. Shaded area: heavily populated savannah country. Inset: map showing position of Trinidad in relation to south and central America.

haematuria was detected only on microscopic examination, the number of red blood cells exceeded 10 per high-power field in the deposit after centrifugation. These patients formed about one-half of those on whom a diagnosis of acute glomerulonephritis was made during the period. Group A streptococci were isolated from 69 of the 133 patients.

From the San Fernando hospital within the same period we obtained streptococci from 38 patients who were subsequently confirmed as cases of acute glomerulonephritis.

The remaining ten cases had occurred in the Port of Spain area between May and August 1965, i.e. before the main part of the investigation was begun.

Uncomplicated skin sepsis in school children

Streptococci were isolated from the skin lesions, and also from nose and throat swabs, of children attending a number of schools. These included cultures obtained in the earlier part of 1965 in three brief school surveys. We carried out three further surveys between September 1965 and October 1966 as part of a general study of the epidemiology of skin sepsis, which will be described in a later publication. Brief details of these investigations follow: in surveys 1–5, nose, throat

and lesion swabs were collected from all children with skin sores, but in survey 6, nose and throat swabs were also collected from healthy children.

1. *Aranguez Schools.* Single visit to two small country schools near Port of Spain in April 1965.

2 and 3. *Princes Town and Mount Pleasant Schools.* Country schools near San Fernando. Swabbing on two occasions in June and July 1965 in connexion with a trial of the antibiotic treatment of skin sepsis.

4. *St Agnes Anglican School, Port of Spain.* Large urban school; single swabbing in November 1965.

5. *Mucurapo Girls' School, Port of Spain.* Urban school; six visits in 2 months, November 1965–January 1966.

6. *St Joseph Presbyterian School.* Small rural school 7 miles east of Port of Spain. Nose and throat swabs from all children weekly, and separate swabs of each skin lesion; April 1966 to April 1967, but only first 27 weeks reported here.

Survey of distribution of serotypes of group A streptococci in Britain 1964–65

A collection of 445 cultures of group A streptococci, isolated in 17 public health laboratories from all classes of streptococcal diseases, forming part of an international survey of the distribution of serotypes of group A streptococci (Parker, 1967), was included for comparison, because these cultures were typed with the same sera as the Trinidad streptococci.

BACTERIOLOGICAL METHODS

Cultural

The Port of Spain and Arima hospitals could be visited as required, and nephritis cases were seen and swabbed as soon as possible after admission. Visits to San Fernando were made no more often than once a week.

Plates of culture medium were always taken to the hospital ward or school, and swabs were inoculated on the medium immediately after the specimen had been taken. The specimens from the San Fernando hospital include some taken by the hospital staff and kept in transport medium (Holmes & Lermit, 1955) until the next visit to the hospital was made.

Cotton-wool swabs were used for the collection of all specimens; they were moistened with sterile physiological saline before sampling the nose or skin lesion.

The culture medium used was 5 % horse-blood agar containing crystal violet in a concentration of 10^{-6}, which was found to be optimal for the batch of dye being used. Cultures were incubated overnight at 37° C. in air.

Representative colonies of β-haemolytic streptococci were subcultured, and grouping was performed in Trinidad by the precipitin method after extraction with acid (Lancefield, 1933) or digestion with *Streptomyces* enzyme (Maxted, 1948).

At least one strain of group A streptococci from each positive culture was sent to Colindale for typing. A loop was charged from a pure culture on a blood agar plate. This inoculum was rubbed on to a sterile filter-paper disk, which was allowed to dry in air. The disk was then placed in a gelatin capsule which had previously

been sterilized by ultra-violet light. On receipt in London, each disk was transferred to a tube of nutrient broth containing 10% horse blood, which was then incubated. Less than 1% of the strains failed to grow.

Typing methods

A combined typing system was used (Williams & Maxted, 1953), in which the streptococci were first examined for T-antigens by slide agglutination and were subsequently tested by the precipitin method with a limited number of M-antisera determined according to the T-typing pattern of the strain.

Agglutination typing

Trypsinized suspensions of streptococci were tested by slide agglutination with antisera for the following T-types: 1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13, 14, 15, 17, 18, 19, 22, 23, 25, 27, 28, 44 and 47, and for the provisional T-types Imp. 19 and B 3264. Antisera of the series 5, 11, 12, 27 and 44 had been specially absorbed by the method of Pakula (1951; see McLean, 1953). During the course of the investigation, a method for the cross-absorption of T-antisera for types 14 and 49 was introduced (Maxted *et al.* 1967), and specially absorbed sera for these types became available. All streptococci which had been agglutinated by the routine T/14 serum were re-examined with the specially absorbed T/14 and T/49 sera.

The above antisera are the ones used in our routine typing of streptococci isolated in Britain, and our experience had been that they usually gave clear-cut results. With the Trinidad streptococci, many cross-reactions were seen; the most common patterns of cross-reaction were between type 49 and members of the 8, 25, Imp. 19 and of the 3, 13, B 3264 agglutination-complex, but a number of other unusual combinations were seen. Further investigation showed that these cross-reacting strains did not absorb agglutinins from all the antisera by which they were agglutinated. It was concluded that they possessed additional trypsin-resistant agglutinating antigens not represented in the current T-typing system. The antibodies to these cross-reacting antigens could be removed by further absorption of the T-typing antisera with Trinidad streptococci which were agglutinated by the sera but did not remove the type-specific antibody from them.

Absorptions of this kind were carried out on the antisera for T-types 3, 13, B3264, 25, Imp. 19, 9, 12, and 49, and these sera were used for the examination of any cultures which gave unusual cross-reactions.

Precipitin typing

Capillary precipitin tests (Swift, Wilson & Lancefield, 1943) were carried out on acid extracts of organisms grown in Todd-Hewitt broth. The following antisera were used throughout the investigation: M-antisera for types 1, 2, 3, 5, 6, 9, 11, 12, 14, 15, 17, 18, 19, 22, 23, 24, 25, 26, 29, 30, 31, 33, 36, 37, 39, 41, 43, 46, 47, 48, 50 and 51; R-antisera for types 3 and 28. In addition, M-antisera for type 49 and for provisional types 52 and 55 became available during the investigation, and were used as will be described.

While this work was in progress, the value of adding Neopeptone to Oxoid

Todd-Hewitt broth also became apparent, particularly in increasing the yield of M-antigens for types 41 and 49 (see Maxted *et al.* 1967; Top, Wannamaker, Maxted & Anthony, 1967). All except some of the earlier cultures were grown in the improved medium, and early cultures having T-agglutination patterns suggesting that they might belong to either of these types were retested after growth in it.

RESULTS

The 1964–6 *epidemic of nephritis in Trinidad*

The course of the outbreak may be followed in the fluctuation of the rate of admission of patients with nephritis to the two main hospitals in Trinidad. The number of children with nephritis admitted each month to the Paediatric Department of the Port of Spain General Hospital is shown in Fig. 2. This curve shows a

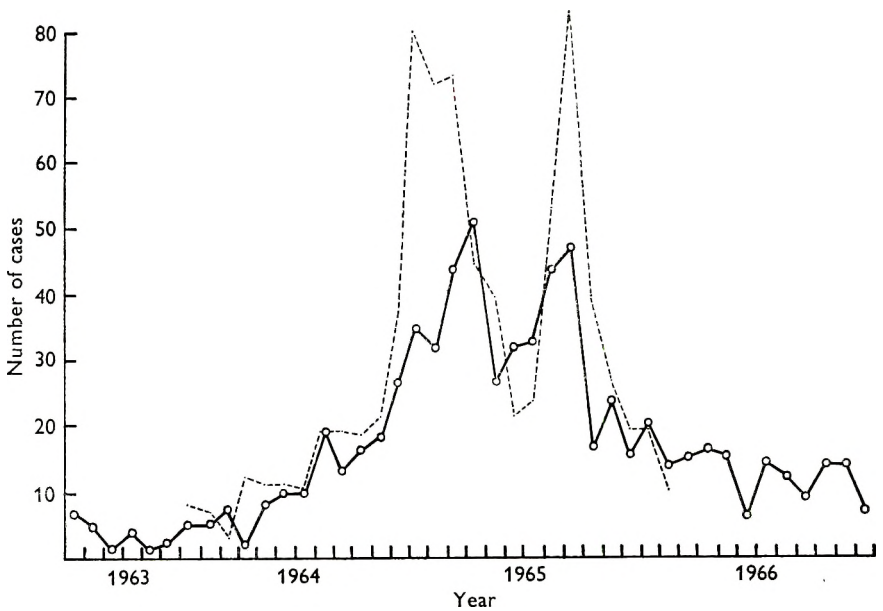

Fig. 2. Number of cases of nephritis admitted each month to two hospitals in Trinidad. O — O, Port of Spain General Hospital (Paediatric Department only). – – –, San Fernando General Hospital (Poon-King, 1968).

striking similarity to that for all admissions with nephritis to the San Fernando General Hospital, which is also shown in the figure (T. Poon-King, personal communication; Poon-King *et al.* 1967). The records of both hospitals show an outbreak extending from mid-1964 until early 1966, and in both there is a striking double peak in the admission rates, with an intervening interval of several months.

After the 1959–60 outbreak (Symonds, 1960) relatively few cases of nephritis were seen in either hospital for several years, and in 1963 admission rates to both were between three and five cases a month. In both hospitals there was some increase in early 1964, and a striking acceleration in the latter months of this year. This was particularly obvious in San Fernando, where the peak of the first wave

of the epidemic occurred in January 1965. The rise continued more gradually in Port of Spain, and the highest rate of admission here was in April 1965.

Admissions to both hospitals fell after April 1965, but rose again in the following August. The second wave of admissions reached its peak simultaneously in the north and the south in September 1965. By the end of the year admissions to both hospitals had fallen to the mid-1964 level.

The first bacteriological observations were made in March 1965, at the height of the first wave of nephritis cases in San Fernando, by Dr Elizabeth Potter, of Northwestern University, Chicago. Our main investigation began in September 1965, at the peak of the second wave, and extended until August 1966, when the main epidemic was over.

Table 1. *Isolation of group A streptococci from patients with acute nephritis admitted to the Port of Spain and Arima hospitals, September* 1964 *to August* 1965

	Total no. patients examined	No. patients with group A streptococci			
		Total	in skin lesion	in nose swab	in throat swab
With skin sores	101	67	64	17	7
Without skin sores	32	2	0	2	0
Total	133	69	64	19	7

Isolation of group A streptococci from cases of nephritis

Group A streptococci were isolated from 69 of the 133 patients in the Port of Spain and Arima hospitals (52 %), as shown in Table 1. Unhealed skin sores were observed on 101 of the patients on admission to hospital (76 %).

Group A streptococci were isolated from 67 of the 101 patients with skin sores (66 %). The streptococcus was present in the sore in 64, in the nose in 17, and in the throat in only seven patients. The organism was isolated only from a skin lesion in 48, from a skin lesion and the respiratory tract in 16, and from the respiratory tract only in three patients.

Very few isolations of group A streptococci were made from patients who did not have skin sores. In two out of 32 patients (6 %) the organism was isolated from the nose, but no streptococci were found in any of the throat swabs. The fact that group A streptococci were so seldom found in the respiratory tract in the absence of a skin lesion supports the view that nephritis did not often follow a primary respiratory tract infection in this epidemic.

Unpublished studies by one of us (D. C. J. B.) of uncomplicated skin sores in school children suggest that lesions not treated with antibiotics have a 'half-life' of about 10 days. It was to be expected, therefore, that in a proportion of patients with nephritis the skin lesion would have healed before admission to hospital.

We also isolated group A streptococci from 38 patients in the San Fernando hospital in whom a diagnosis of acute nephritis was subsequently confirmed. Since swabs were obtained at weekly intervals, some of them were collected after the patients had been in hospital for several days. The organism was isolated from a skin lesion in 28 of these patients.

Typing of group A streptococci from case of nephritis

Group A streptococci were isolated from 172 swabs from 117 patients with nephritis: this total of 117 includes the 10 patients mentioned on page 658 from the Port of Spain area from whom streptococci were isolated between May and August 1965. Two or more positive swabs were obtained from 39 patients; the streptococci isolated from different sites had the same T-typing pattern in 32 patients, but seven patients yielded serologically distinguishable streptococci from different swabs. In addition, two different streptococci were isolated from the same swab on one occasion. We therefore had 125 serologically distinct streptococci from patients with nephritis.

Table 2 summarizes the T-typing patterns of these distinct strains, and also of the 445 cultures from all classes of streptococcal illness in Britain, which had been typed with the same antisera.

Four distinct T-typing patterns together accounted for 97 % of the Trinidad nephritis strains. The most common of these was agglutination by the routine T/14 antiserum, and by the specially absorbed T-antiserum for type 49. The evidence that this antigen is characteristic of the Red Lake streptococcus, which has been found in association with nephritis following skin sepsis, has already been presented by Maxted and his colleagues (1967). Although this organism formed 38 % of the strains isolated from cases of nephritis in Trinidad, members of the 8, 25, Imp. 19 T-complex (29 %) and of the 3, 13, B 3264 T-complex (21 %) appeared not much less frequently. Only two cultures were agglutinated by the T/12 antiserum. It therefore appeared that type 12, which is the type most frequently associated with nephritis following streptococcal infection of the respiratory tract, was of little importance as a cause of nephritis in Trinidad.

The most remarkable finding, however, was our almost total failure to detect any of the M-antigens commonly found in British group A streptococci. With the regular set of precipitating sera used in our laboratory, an M-antigen was detected in only one of the 125 cultures—a member of M-type 18. With the same set of sera, M-antigens were detected in 32 % of the British survey strains. This difference is apparent even when we compare strains with similar T-typing patterns. For example, members of the 3, 13, B 3264 complex isolated in Britain include about one-third which have the M-antigen 3, but this antigen has never been identified in Trinidad.

The Trinidad nephritis streptococci resemble in T-typing pattern and in apparent absence of M-antigen the streptococci isolated in Europe and North America from cases of superficial skin sepsis. The association of members of the 3, 13, B 3264 and of the 8, 25, Imp. 19 T-complex with impetigo was noted in Britain some years ago (Parker, Tomlinson & Williams, 1955), but nephritis secondary to skin sepsis was not observed. At Red Lake, Minnesota, organisms with the 3, 13, B 3264 T-pattern were predominant in skin sores at a time when nephritis was absent (Anthony, Perlman & Wannamaker, 1967*b*). In Alabama, on the other hand, organisms with the agglutination pattern 25/Imp. 19 were the largest group to be found in association with impetigo and nephritis, and

Table 2. *Typing of group A streptococci from cases of nephritis in Trinidad*

(The 172 cultures isolated from 117 patients included 125 distinct strains recognizable by T-agglutination. The results of typing 445 cultures isolated from all classes of streptococcal disease in Great Britain are included for comparison.)

Agglutinated by T-antiserum for types	Trinidad: No. distinct strains from cases of nephritis: Port of Spain, May–Aug. 1965	Port of Spain and Arima, Sept. 1965–Aug. 1966	San Fernando, Sept. 1965–Aug. 1966	Total	Great Britain (all streptococcal diseases)
1	0	0	0	0	31 (7%)
2	0	0	0	0	4 (<1%)
3, 13, B 3264	3	17	6	26 (21%)	63 (14%)
4, 4/28	0	9	2	11 (9%)	89 (20%)
5, 27, 44	0	1	0	1 (<1%)	21 (5%)
6	0	0	0	0	15 (3%)
9	0	1	0	1 (<1%)	8 (2%)
11	0	0	0	0	13 (4%)
12	1	1	0	2 (2%)	132 (30%)
14 (14)	0	0	0	0	2 (<1%)
14 (49)	2	27	18	47 (38%)	
18	1	0	0	1 (<1%)	0
22	0	0	0	0	12 (3%)
8, 25, Imp. 19	4	20	12	36 (29%)	27 (6%)
28	0	0	0	0	10 (2%)
Other or untypable	0	0	0	0	18 (4%)
Total no. distinct strains	11	76	38	125	445
No. cultures M-positive*	**1**	**0**	**0**	**1**	**142 (32%)**

* Before introduction of M-antisera for Types 49 and 55.

Table 3. *Distribution of T-agglutination patterns among group A streptococci isolated from skin lesions, and from the nose and throat swabs of children with skin lesions, in five Trinidad schools*

Agglutinated by T-antiserum for types	No. of distinct strains: From cases of uncomplicated skin sepsis: Aranguez, May 1965	Mount Pleasant, July–Aug. 1965	Princess Town, July–Aug. 1965	St Agnes, Nov. 1965	Mucurapo, Nov. 1965–Jan. 1966	Total	From acute nephritis
3, 13, B 3264	4	3	20	11	14	52 (27%)	26 (21%)
4 or 4/28	0	1	1	1	2	5 (3%)	11 (9%)
5, 27, 44	1	1	8	1	1	12 (6%)	1 (<1%)
9	0	1	1	3	6	11 (6%)	1 (<1%)
11	1	0	0	1	8	10 (5%)	0
12	3	1	1	7	14	26 (13%)	2 (2%)
8, 25, Imp. 19	2	0	14	6	15	37 (19%)	36 (29%)
49	2	7	7	8	5	29 (15%)	47 (38%)
Other	1	0	0	6	4	11 (6%)	1 (<1%)
Total no. distinct strains	14	14	52	44	69	193	125

there outnumbered members of type 49 (Dillon, Moody, Maxted & Parker, 1967). The investigation at Red Lake (Top *et al.* 1967) provided an explanation of the apparent absence of M-antigen from skin streptococci, when it was found that the common 3, 13, B 3264 T-complex contained, in addition to M-types 3, 13, and 33, others such as M-type 41 and the two new provisional M-types 52 and 53.

It appeared, therefore, that our lack of success in finding M-antigens might be due to the presence there of hitherto undescribed M-types, or of types for which we had not succeeded in making a good precipitating antiserum. While the investigation was in progress, we were able for the first time to make a reasonably potent M-antiserum for type 49 (Maxted *et al.* 1967). All streptococci that had been agglutinated by the special T-antiserum for type 49 were tested with this serum, and nearly half of them (26 of 60) gave a precipitin reaction. Since this time we have made better M-antisera for this type, and nearly all the T/49 streptococci isolated in Trinidad in 1967 have been found to be M-positive.

Since type 49 streptococci formed little more than one-third of the strains isolated from cases of nephritis in Trinidad, we were encouraged to look among the other streptococci for possible nephritogenic strains. To narrow the field of search, we first compared the T-typing patterns of streptococci from cases of nephritis with those of streptococci from children with skin sepsis not complicated by nephritis.

Typing of group A streptococci from school children with skin sepsis

Five sets of cultures of group A streptococci, isolated from the skin lesions and from the nose and throat swabs of children with skin sepsis were collected between April 1965 and January 1966, while the nephritis outbreak was in progress. The result of T-typing these cultures is set out in Table 3, which shows the number of distinct strains with each T-typing pattern; as in Table 2, multiple isolations of the same strain from a patient appear only once. The distribution of T-typing patterns among strains from each school is shown separately, and the percentage-distribution of T-typing patterns in the whole series is compared with that found in the streptococci from cases of nephritis.

Streptococci of many different serotypes were present in each of the schools, and the variety of T-typing patterns was considerably greater than in the cultures from cases of nephritis. Several organisms poorly represented in the nephritis series (e.g. T/12, T/11, T/9 and the 5, 27, 44 T-complex) were found frequently among the school children, and were at times prevalent in individual schools.

Type 49 streptococci were found much less often in uncomplicated skin sepsis (15 %) than in nephritis (38 %) and this supported the view that they were nephritogenic. A similar comparison suggested that there might be a nephritogenic strain in the 8, 25, Imp. 19 complex, in that 29 % of nephritis strains, but only 19 % of strains from patients with uncomplicated skin sepsis had patterns in this complex. There was no such indication for the 3, 13, B3264 strains.

Further investigation of the 8, 25, *Imp.*19 *T-agglutination complex*

In Trinidad, as in Alabama, most of the streptococci belonging to the 8, 25, Imp.19 complex were agglutinated only by the T-antisera 25 and Imp.19. A culture with the T-typing pattern 25/Imp. 19, isolated from the skin lesion of an undoubted case of nephritis admitted to San Fernando General Hospital in October 1965 [C. Chudee, No. 65/4127] was therefore used to prepare an M-antiserum in a rabbit. Precipitating antibody was formed promptly; the serum gave a strong reaction with an extract of the homologous strain, but not with extracts of the type strains of M-types 1 to 51. It also enhanced the bactericidal effect of fresh whole blood on the homologous strain, but not on members of other M-types. It therefore appeared to possess a new M-antigen.

The serum was incorporated in our routine set, and was used in the typing of many streptococci from Trinidad from March 1966 onwards, but no positive results were obtained. Interest in the serum therefore waned, because similar situations had been observed before on a number of occasions.

In 1967, Dr Potter sent us a culture of an organism described as 'Trinidad A', together with a sample of antiserum prepared with it. She told us that many of the organisms she had isolated in Trinidad in March 1965 appeared to belong to a new M-type of which the culture 'Trinidad A' was representative, and she made available to us the information she has since published about the new type (provisional M-type 55; Potter *et al.* 1967).

A comparison of the M-antigen of our strain no. 65/4127 and of 'Trinidad A' (provisional Type 55) showed that they were identical. We therefore re-examined nearly all of our early collection of 25/Imp. 19 strains from Trinidad with the serum prepared with culture no. 65/4127. It then became apparent that M-type 55 was indeed associated with a number of cases of nephritis, but only in the earlier part of the outbreak.

Table 4. *Number of cases of acute nephritis associated with group A streptococci of type* 49, *of provisional type* 55, *and with other types of group A streptococci*

No. of cases	Mar. 1965*	May–Aug. 1965	Sept.–Dec. 1965	Jan.–Apr. 1966	May–Aug. 1966	Total
Type 49	1	2	16	13	16	48
Type 55	11	3	9	0	0	23
Other types only	4	5	13	16	24	62
Total no. cases	16	10	38	29	40	133

* Data provided by Dr E. V. Potter

The re-examination of these cultures greatly clarified the picture of the epidemic. Table 4 was constructed by combining our results with those of Dr Potter, and shows the number of patients admitted to hospital with nephritis within certain periods of time from whom type 49, provisional type 55, and other types of group A streptococci, were isolated.

Dr Potter identified provisional type 55 among the group A streptococci from

11 of 16 patients with nephritis in San Fernando in March 1965, but found type 49 in only one patient. The few cultures isolated in Port of Spain between May and August 1965 included members of both types. In the period September to December 1965, type 49 was for the first time the most common type, but a considerable minority of provisional type 55 was present. From January 1966 onwards, no culture of provisional type 55 was isolated, and type 49 was by far the commonest type present.

A consideration of Table 4 in conjunction with Fig. 2 indicates that provisional type 55 predominated at the time of the first peak of the epidemic, and that it was progressively replaced by type 49 during the second wave of cases.

Re-examination of 25/Imp. 19 streptococci from the cases of uncomplicated skin sepsis revealed few streptococci of provisional type 55. Thirty-seven strains with this T-pattern were isolated from the children in the five school surveys (Table 3); 33 were re-examined and only two members of provisional type 55 were found. There were no members of this M-type among the many 25/Imp. 19 streptococci from other sources in Trinidad examined during 1966 and 1967.

While this work was in progress, Dillon, Reeves & Maxted (1968) re-examined the 25/Imp. 19 streptococci that had been isolated from cases of nephritis in Alabama, and found that many of them had the M-antigen 2. With rare exceptions, streptococci with this unusual combination of M- and T-antigens were found only in patients with nephritis and their close associates. This unexpected finding caused us to re-test with every available antiserum all the cultures from Trinidad that belonged to the 8, 25, Imp. 19 T-complex and gave no precipitin reaction with the M-antiserum type 55. Good antisera for M-types 2 and 25 were included, but no M-antiserum for type 8 was available. None of the cultures gave a precipitin reaction with any of the antisera.

Multiple infections with different types of streptococci

If both provisional type 55 and type 49 are responsible for nephritis in Trinidad, a cause was established for 71 out of 133 cases (54 %; see Table 4). What then are we to think of the significance of the streptococci isolated from the remaining 46 % of cases?

It is clearly unreasonable to assert that every strain of group A streptococci isolated from a patient with acute nephritis was the cause of this disease. We had observed that seven out of 39 patients from whom two or more isolations of streptococci had been made on admission to hospital (18 %) were carrying two serologically distinct streptococci at different sites. In populations with a high incidence of streptococcal infection, particularly when there are opportunities for heavy dispersion of streptococci from exposed skin lesions, the frequent transfer of infection by the skin-to-skin route might lead to a high rate of multiple infection.

Indeed, Anthony *et al.* (1967 *b*), who typed serial cultures of group A streptococci from the skin lesions of children in the Red Lake Indian Reservation, found that, when two or more isolations were made from the same patient, over half of the patients were found to be carrying more than one distinct type of streptococcus. These observations, however, were spread over several months, and it is

not possible to obtain from the published information any idea of the rate of 'change' of type.

We therefore studied the frequency of multiple infection at St Joseph Presbyterian School, where a continuous study of skin sepsis was in progress. This was a rural school in north Trinidad in which there was a relatively high rate of streptococcal infection. Weekly swabbing was carried out during term-time for 27 weeks from the beginning of April 1966. Nose and throat swabs were collected from each child, and each unhealed skin lesion was swabbed separately. In any one week, on average 26 % of the children had skin lesions, and group A streptococci were isolated from skin lesions of 15 % of the children. The average throat carrier-rate was 6 %, and the nasal carrier-rate was 4 %.

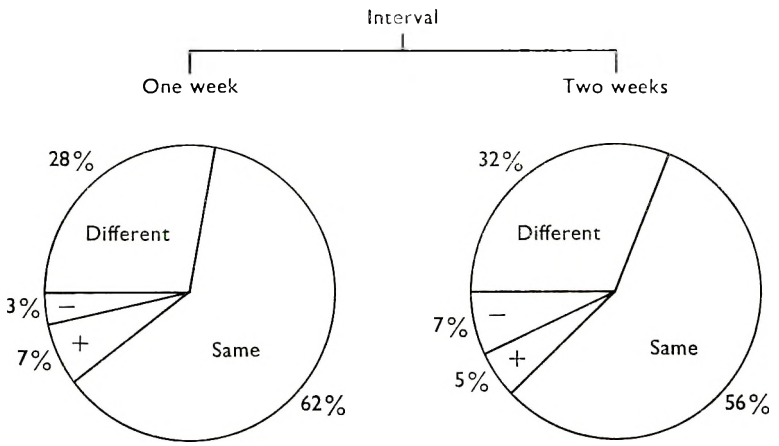

Fig. 3. Isolation of serologically distinct strains of group A streptococci from the same person on two occasions. Comparison of the T-typing patterns of the strains when group A streptococci were isolated on both occasions at an interval of (*a*) 1 week and (*b*) 2 weeks. Percentage of occasions on which: (1) strains with the same T-typing pattern were isolated on both occasions ... ('same'); (2) all strains isolated on the first occasion were different from those isolated on the second ('different'); (3) the original strain persisted, and a fresh one was added ('+'); (4) one of the two strains present on the first occasion was absent on the second ('−').

One colony from each positive culture was typed, and a comparison was made of the T-typing patterns of streptococci isolated from the same patient at different sites on the same day, and on successive weekly examination. During 27 weeks' observation, 84 % of the 98 children had one or more streptococcal infection; 56 % had two or more infections due to serologically distinct streptococci, 26 % had three or more infections, 9 % had four or more infections, and 2 % had five infections. Group A streptococci were isolated 108 times from two or more swabs collected from the same patient on the same day, and more than one type of streptococcus was found 24 times (22 %).

Next, all occasions on which group A streptococci were isolated from one patient on two successive weeks were examined (Fig. 3). The same type of streptococcus was found both times on 62 % of occasions; but on 28 % of occasions, the strain

found the first time was not found the second time, and an entirely different strain was present. In a further 7 % of occasions the original strain persisted but a new one was added, and in 3 % of occasions one of two strains had disappeared after a week. When the interval between positive swabs was 2 weeks, the percentage 'change' of type was slightly but not greatly increased.

These findings suggest that an attempt to identify the type of streptococcus responsible for the primary skin infection which initiated an attack of nephritis by the examination of swabs collected when the patient is admitted to hospital will give an incorrect answer in at least one case in three. This is probably an underestimate of the error, because it is based on the results of T-typing, and organisms considered alike on the basis of T-agglutination patterns may have different M-antigens.

The finding that the two types of streptococci suspected of nephritogenic properties could be isolated from only 54 % of cases is therefore consistent with the view that they were responsible for most of the cases of nephritis in the recent outbreak in Trinidad.

DISCUSSION

Since the early years of this century it has been recognized that acute glomerulonephritis may sometimes follow streptococcal infections of the skin (Dillon, 1967). Recently it has become apparent that in parts of north and central America most cases of glomerulonephritis are associated with impetiginous skin lesions.

The difficulties that have been encountered in recognizing nephritogenic strains of group A streptococci from skin lesions are attributable in part to the characters of the organisms, and in part to the frequency with which more than one serotype of streptococcus can be isolated from the lesions of one patient. In all investigations, however, there has been a notable absence of streptococci of M-type 12, which is responsible for most cases of nephritis which follow infections of the upper respiratory tract (Rammelkamp, Weaver & Dingle, 1952).

For some time it has been recognized that group A streptococci from skin lesions seldom have M-antigens that are detectable with the antisera used in routine typing (Parker *et al.* 1955; Barrow, 1955; Dillon *et al.* 1967). Top *et al.* (1967) have shown that this is not because 'skin' streptococci lack M-antigens, but because they belong to previously unrecognized or supposedly rare M-types.

It was not surprising, therefore, that the first nephritogenic 'skin' streptococcus to be identified should have been a member of a hitherto undescribed M-type, now known as type 49 (Updyke, Moore & Conroy, 1955). This type was first found in the Red Lake Indian Reservation, Minnesota, in 1953 (Kleinman, 1954), but was rarely recorded elsewhere during the next 10 years, because of a widespread lack of success in making sufficiently potent M-antisera for the type. There is now evidence that it is to be found, and has caused nephritis, over a wide geographical area (Maxted *et al.* 1967).

Two outbreaks of nephritis due to type 49 have occurred at Red Lake, separated by an interval of 13 years during which the disease was rarely seen

(Anthony *et al.* 1967 *a*). Skin lesions were prevalent during the intervening period, but the group A streptococci isolated from them were of different types (Anthony *et al.* 1967*b*; Top *et al.* 1967).

In at least two other areas in which type 49 has been shown to be an important cause of nephritis, there is evidence that it is not the only nephritogenic streptococcus present in skin lesions. In Birmingham, Alabama, nephritis and streptococcal skin sores are prevalent each year in the summer and early autumn; over 80% of the cases of nephritis, and over 90% of skin sores occur in the months July to September. Members of M-type 49 make up about a quarter of the streptococci from the skin lesions of cases of nephritis (Dillon *et al.* 1967). Further investigations showed that a larger proportion (46%) were an unusual streptococcal strain which had the M-antigen 2, but with the agglutination pattern 25/Imp. 19. Streptococci with this antigenic structure were rarely found except in cases of nephritis or their family contacts (Dillon *et al.* 1968).

In Trinidad, major epidemics of nephritis occurred in 1952–4, in 1958–9, and in 1964–6. Little is known about the first epidemic, which was revealed by a retrospective examination of the records of the San Fernando General Hospital (Poon-King *et al.* 1967), but the two later outbreaks appear to have involved the whole island. Our initial observations, made in the second half of the 1964–6 epidemic, revealed that some 39% of the group A streptococci from cases of nephritis belonged to M-type 49. However, in a small collection of streptococci isolated several months earlier, Dr Potter found few members of M-type 49, but a predominance of cultures with the T-agglutination pattern 25/Imp. 19, which proved to have a new M-antigen of provisional type 55 (Potter *et al.* 1967). Retrospective examination revealed that a number of M-type 55 streptococci were present in the earlier part of our collection of cultures. A consideration of our results with those of Dr Potter suggested that in March 1965, at the height of the first wave of nephritis cases, provisional type 55 was the predominant strain, and that it became progressively less common between May and December 1965, and then disappeared; type 49 was seldom seen in April 1965, but exceeded provisional type 55 in frequency at the peak of the second wave, and was the only supposedly nephritogenic strain isolated in the stage of decline of the epidemic. From this, it appears that the double-peaked outbreak consisted of two partly overlapping epidemics due to different serotypes.

At the outset of the investigation in Trinidad, very few of the group A streptococci from cases of nephritis were typable with the available M-antisera, and T-typing was the only available means of characterizing them. This proved difficult, because cross-reactions were unusually frequent with the regular set of T-typing sera, and had to be removed by a series of additional absorptions. It then became apparent that the streptococci included strains with several different T-typing patterns. When the relationship between the T-antigens of types 14 and 49 had been elucidated, a highly specific T-agglutinating serum for type 49 was obtained and this type was found to be the most common streptococcus in our series of cultures from cases of nephritis. This finally was confirmed later when we succeeded in making a sufficiently good M-antiserum for type 49.

The remaining strains from nephritis cases showed a predominance of members of the 3, 13, B 3264 and the 8, 28, Imp. 19 agglutination complexes, which are known to contain members of several M-types, together with a large number of strains in which no M-antigen has yet been identified. Some guesswork was therefore necessary in deciding which of these strains should be selected for further investigation. The distribution of T-typing patterns among the streptococci from cases of nephritis and from children without nephritis led to a presumption that the 8, 25, Imp. 19 complex might contain a nephritogenic strain. By good fortune, we and the Northwestern University group both chose a member of provisional type 55 for serum production, and our results are therefore comparable. Between us, we succeeded in finding a probable cause for just over half of the cases of nephritis from which a streptococcus was isolated.

We had to consider the possibility that some of the other streptococci isolated from cases of nephritis were also nephritogenic. Very few of them, or indeed of the streptococci isolated from skin lesions unassociated with nephritis, or from other streptococcal diseases in Trinidad, had M-antigens identifiable with existing antisera. A careful search for M-antigen 2 in strains with the T-agglutination pattern 25/Imp. 19 failed to reveal a single culture resembling the Alabama strain with this antigenic structure. There is a suggestion in Table 4 that the proportion of cases of nephritis associated with one or other of the supposedly nephritogenic types was falling towards the end of the period of observation, and further investigations in 1967 confirmed this trend. Indeed, by mid-1967, when the incidence of nephritis had again risen above the immediate postepidemic level, type 49 had become uncommon, and the most frequently encountered strain had the T-agglutination pattern 25/Imp. 19, but appeared to have no recognizable M-antigen. An antiserum made with one such strain [V. Ramkissoon, no. 67/3890] was found to contain antibody to a hitherto unrecognized M-antigen (now designated provisional type 57). About two-thirds of the T-25/Imp. 19 cultures from nephritis cases in the second half of 1967 gave a precipitin reaction with this serum. These results will be published in detail later.

Patients with skin lesions frequently harbour two or more different strains of group A streptococci, and this makes it difficult to identify nephritogenic strains with certainty unless they are responsible for clear-cut localized epidemics, as at Red Lake. Our calculations (Fig. 3) of the probable rate of 'change' of type suggests that in Trinidad only about two out of every three of the streptococci isolated from cases of nephritis on admission to hospital are likely to have been the ones responsible for the nephritis. This estimate is only approximate, and is based on the assumption that the predominant streptococcus in each lesion was the only one present. This may not be so, though we found two different streptococci in the same lesion on only one out of 150 occasions on which we typed ten different colonies from the primary plate. Infection with multiple types was assumed when streptococci with different T-agglutination patterns were isolated from the same patient; therefore any lack of reproducibility in the typing would have exaggerated its frequency. Despite the manifest difficulty in T-typing the Trinidad strains, we do not think that this occurred often enough to influence

our general conclusions. Multiple streptococcal infection of skin lesions has been reported elsewhere, but it appears to have been exceptionally common in Trinidad. The fact that *Hippelates* flies are very commonly to be seen feeding from the skin lesions, and will deposit group A streptococci on a blood agar plate several hours after such a meal (Bassett, 1967), suggests that the passive transmission of the organism may be an important factor in causing multiple infections.

The periodicity of nephritis epidemics in Trinidad is not explicable on the basis of the present evidence. An epidemic in a small and relatively isolated community such as the Red Lake Indian Reservation might follow the introduction of a nephritogenic strain of streptococcus for which there is little type-specific immunity in the child population. Alternatively, a rise in the incidence of nephritis might be a consequence of an increase in the total number of skin infections, if nephritogenic 'skin' streptococci are constantly present in the population. This appears to be the situation in Alabama. In Trinidad, skin lesions due to group A streptococci are present at all seasons of the year, and affect at least 10% of the child population of the island at any one time. There have been long periods of relative freedom from nephritis, and large epidemics have occurred at intervals of about 6 years. However, the most recent epidemic appears to have consisted of two distinct outbreaks in rapid succession, which were observed at about the same time in the northern and southern parts of the island. It is remarkable that the two nephritogenic streptococci appear to have spread so rapidly and evenly through the relatively immobile child population of the villages of Trinidad. The regular intervals between the major outbreaks might suggest that type-specific immunity to nephritogenic strains plays some part in preventing epidemics, but it would be necessary to assume that immunity to type 49 and to provisional type 55 in the child population had fallen below a critical level at about the same time.

Investigations over limited periods of time in three geographical areas have produced evidence of nephritogenic strains in three different M-types of group A streptococci. Two of these strains have so far been incriminated only in a single area, though they have not yet been sought widely elsewhere. There is evidence, however, that M-types found frequently in skin lesions in one area are rare in others. For example, streptococci of M-types 41 and 52, with the T-agglutination pattern 3/13/B 3264, were very prevalent at Red Lake (Top *et al* 1967). We have searched extensively for them among streptococci with similar T-patterns from Trinidad and Britain, but have very rarely found them. The number of unidentified M-types among 'skin' streptococci is probably still quite considerable.

SUMMARY

The recent epidemic of acute glomerulonephritis in Trinidad had two peaks, separated by an interval of about 6 months.

Evidence is presented that there were in fact two successive but overlapping epidemics, the first due to streptococci of provisional M-type 55, and the second to streptococci of M-type 49.

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