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A six-year study of coxsackievirus B infections in heart disease

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(*Received* 14 *February* 1974)

SUMMARY

Virological examination of 385 patients with suspected heart disease and 26 with Bornholm disease over a period of 6 years suggested that Coxsackie group B virus infections were associated with at least half the cases of acute myocarditis and one third of the cases of acute non-bacterial pericarditis. Complement-fixation tests revealed only a few cardiac illnesses associated with other infections (influenza and *Mycoplasma pneumoniae*). No evidence of infection was found in chronic cardiac disease.

INTRODUCTION

Although viruses of Coxsackie group B have been those most commonly implicated in heart disease, good evidence as to their causative role is hard to find. The problem arises partly from difficulties in establishing the specific virological diagnosis. Since these viruses can be isolated from the throat for a few days, from faeces for a week or two (longer in children), or from pericardial fluid or myocardial biopsy tissue, virus isolation provides the quickest and simplest method of virological diagnosis. Often, however, the infection may have been present long enough for virus excretion to have ceased by the time faeces have been collected for testing, and few cases are seen early enough for virus to be detected in the throat. Less is known about the duration of infection in pericardial fluid or myocardium, but these specimens are rarely available. Serological tests of paired sera provide an alternative method of diagnosis, but are expensive and not technically simple. Detection of a fourfold or greater rising titre of neutralizing antibodies to one or more of the six viruses provides clear evidence of infection, but again the first blood has often been taken too late for the rise to be demonstrable. Since coxsackievirus infections are common and most persons possess antibodies from past infection, in the absence of a rising titre it becomes necessary to attempt to interpret the significance of the particular titres observed: the higher the titre, the greater the probability of recent infection, but the variable response of different individuals prevents certainty in the interpretation of the titres.

These tests have been applied to specimens from patients with suspected cardiac disease received in this laboratory for many years. Since the beginning of 1966, the introduction of an economical microneutralization test for antibodies allowed a more liberal attitude to the testing of sera (Bell & Grist, 1970). Preliminary reports of our findings have appeared elsewhere (Bell & Grist, 1972; Grist, 1972;

Bell & Grist, 1973). The present paper correlates the virological and serological findings over the period 1966–71 with the clinical diagnoses of the patients studied.

MATERIALS AND METHODS

The virological methods and sources of specimens and clinical information were described previously (Bell & Grist, 1970). It must be emphasized that our clinical classification of patients was based on information provided by many different clinicians when submitting specimens, supplemented by standard follow-up enquiry and where necessary by further correspondence. Patients were allocated to one of five groups: (*a*) Bornholm disease, without cardiac features; (*b*) acute non-bacterial myocarditis, including cases with evidence of both myocarditis and pericarditis; (*c*) acute non-bacterial pericarditis; (*d*) other cardiac diseases; (*e*) non-cardiac disease investigated because of initial suspicion of cardiac disease. This classification sufficed for our analytical purposes but cannot provide a detailed clinical profile of the illnesses associated with coxsackievirus infections.

RESULTS

Table 1 summarizes the findings in the different diagnostic groups according to the results of virological tests. Seven virological categories are distinguished – a finer breakdown than was used for the smaller figures in our preliminary reports (Bell & Grist, 1973). Virological categories I-IV carry the higher diagnostic significance and include 65 % of cases of Bornholm disease, 49 % of myocarditis, 30 % of pericarditis, and 10 % of other cardiac and non-cardiac disease groups (Table 2). Antibody titres of 128 (category V) and less (category VI) carry no diagnostic significance and are predominantly associated with 'other cardiac' and non-cardiac illnesses in the series. The relation between diagnostic groups and virological categories are illustrated in another way in Fig. 1 which shows the distribution of diagnoses associated with each virological category. Of patients in category I (virus isolation plus rising antibody titre) 64 % had Bornholm disease, which is known to be caused by coxsackieviruses or occasionally by echoviruses, the associated viruses being Coxsackie B type 2 (two cases), 3 and 4, echovirus type 6 (two cases) and 19; 27 % had acute myocarditis (Coxsackie A1, B2, B3); the only other patient in this group had rheumatic fever and carditis associated with echovirus 19 infection and ASO titre of 833. Almost equally strong evidence of current or very recent infection is provided by categories II and III, which are distributed similarly between diagnostic groups and are combined in Fig. 1: only 9 % of these patients had Bornholm disease but 44 % had acute myocarditis and 26 % acute pericarditis. Titres of 256 (category IV) provide probable but less certain evidence of recent infection, and the diagnostic profile of this category is shifted even further to the right in Fig. 1, intermediate between the previous categories and the non-significant categories V and VI which have been combined in the Figure. Category VII contained very few cases and was associated with all diagnostic groups except Bornholm disease; the virus isolations unsupported by serological evidence indicated faecal excretion but gave no indication of the duration or pathogenic signifi-

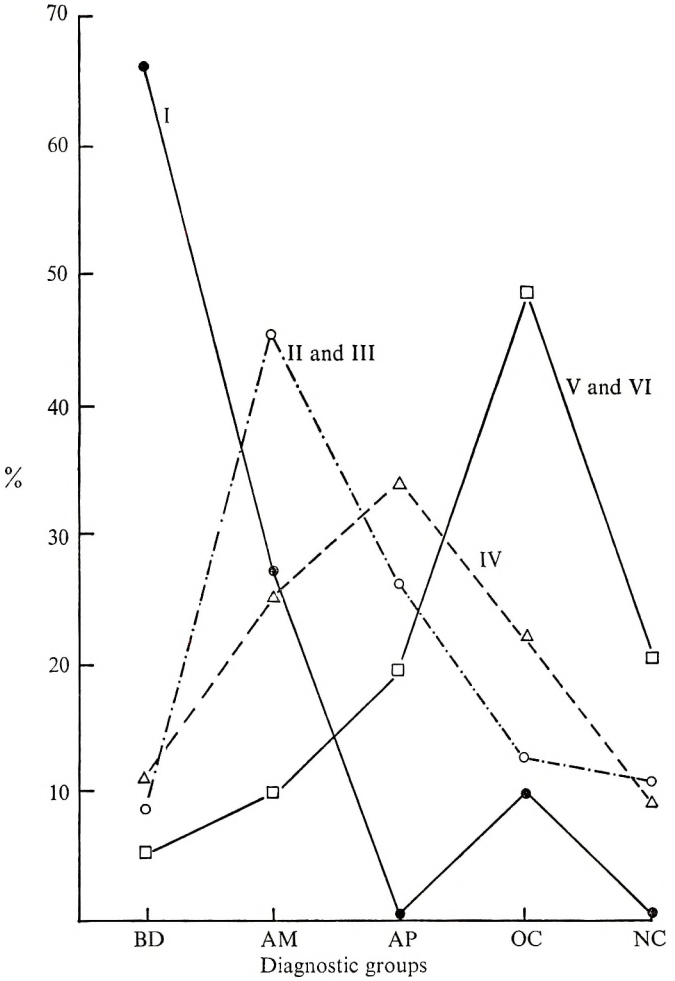

Fig. 1. Distribution of diagnostic groups among virological categories I–VI (see Table 1). BD, Bornholm disease; AM, acute myocarditis; AP, acute pericarditis; OC, other cardiac disease; NC, non-cardiac disease.

Table 1. *Distribution of patients by clinical diagnosis and virological category*

	Virological category							
Diagnostic group	Virus isolation and rising ab.* titre (I)	Rising or falling ab. titre (II)	ab. titre ⩾512 (III)	ab. titre 256 (IV)	ab. titre 128 (V)	ab. titre ⩽64 (VI)	Virus isolation only (VII)	Totals
Bornholm disease	7	2	2	6	2	7	0	26
Acute myocarditis	3	7	12	11	8	24	2	67
Acute pericarditis	0	3	8	15	12	47	1	86
Other cardiac disease	1	1	4	10	18	125	1	160
Non-cardiac disease	0	0	4	3	8	56	1	72
Totals	11	13	30	45	48	259	5	411

* ab. = antibody.

Table 2. *Percentages of virologically 'positive' (categories I–IV) cases in each year of study*

Diagnostic group	Positive in years indicated (%)						No.		
	1966	1967	1968	1969	1970	1971	Tested	Positive	(%)
Bornholm disease	0	67	86	67	50	40	26	17	(65)
Acute myocarditis	0	67	56	44	20	73	67	33	(49)
Acute pericarditis	0	9	23	37	9	56	86	26	(30)
Other cardiac and non-cardiac diseases	0	0	5	16	10	11	232	23	(10)
Total tested	24	35	76	99	74	103	411	99	(24)

Table 3. *Numbers and proportions of virologically 'positive' cases grouped by age and diagnosis*

Diagnostic group	Age-groups (years)			
	−10	−30	−50	51+
Bornholm disease	5/5* (100%)	8/11 (73%)	4/8 (50%)	0/2 (0%)
Acute myocarditis	6/11 (55%)	10/21 (48%)	12/23 (52%)	5/12 (42%)
Acute pericarditis	3/4 (75%)	5/23 (22%)	11/31 (35%)	7/27 (26%)
Other cardiac and non-cardiac diseases	3/13 (23%)	7/51 (14%)	8/86 (9%)	5/80 (6%)
Totals†	17/33 (52%)	30/106 (28%)	35/148 (24%)	17/121 (14%)

* Number 'positive' (categories I–IV)/total in group.
† Ages of 3 patients are not known.

cance of this – in fact, the types of virus were such as to suggest that most were coincidental and irrelevant to the associated illnesses: in myocarditis, echovirus 22 and poliovirus 3; in pericarditis, echovirus 8; in 'other cardiac disease' (Wolf–Parkinson–White syndrome with tachycardia), coxsackievirus A8; in non-cardiac disease, echovirus 30 (probably the cause of the aseptic meningitis in this case). The same rank order of virological positivity was shown by the diagnostic groups in each year of the investigation, with trivial exceptions (Table 2).

In order to evaluate the possibility of bias from unequal age-distribution in the diagnostic groups, the virological results were analysed by diagnosis and age-group. Table 3 shows that the frequency of infection was, as expected, highest in the youngest and lowest in the oldest patients, but in each age-group evidence of infection was more frequent in acute myocarditis and pericarditis than in other conditions (except Bornholm disease).

Table 4 shows the findings in cases classified as 'other cardiac diseases'. The same criteria of virological positivity have been used (categories I–IV). In none of these illnesses did the percentage with evidence of infection significantly exceed that in non-cardiac disease (7/72 = 10%); all three cases of acute rheumatic carditis had elevated ASO titres indicating streptococcal aetiology.

Although not part of the planned study, many of the sera were also tested by complement fixation with other antigens for routine diagnostic purposes. In view

Table 4. *Virological classification of cardiac cases other than acute myocarditis and pericarditis*

Diagnostic group	No. tested	No. 'positive' (virological categories I–IV)
Acute myocardial infarction	48	7 (15 %)
Acute rheumatic carditis	14	3 (21 %)
Other acute diseases	12	1 (8 %)
Myocardial ischaemia	46	1 (2 %)
Other chronic diseases	40	4 (10 %)

Table 5. *Results of complement fixation tests for other infections*

Antigens	Numbers of patients tested per diagnostic group (numbers 'positive'* in parentheses)		
	Acute myocarditis	Acute pericarditis	Other cardiac diseases
Influenza A	34 (1)	44 (1)	74
Influenza B	31	37	71
Influenza C	22 (1†)	25	52
Adenovirus	39 (1)	51	88
Chlamydia group	47	68	124
R. burneti	50	69	131
M. pneumoniae	36 (1†)	50 (1)	85 (1)

* Titres $\geqslant 256$ or fourfold or greater rising or falling titres.
† Same case.

of interest in the possible aetiological significance of other viruses and mycoplasmas in cardiac disease, the main results are summarized in Table 5. The few serologically significant results were as follows:

Case 1. Forty-five-year-old woman diagnosed 'acute myocarditis': paired sera gave falling influenza A antibody titres of 32, < 8 indicating recent infection; Coxsackie B and other serological tests negative.

Case 2. Forty-six-year-old woman diagnosed 'influenza with acute pericarditis': paired sera gave rising influenza A antibody titres of 32, 512+; other complement fixation tests negative, but significant titres (256, 256) of coxsackievirus B1 and B2 neutralizing antibodies.

Case 3. Twenty-eight-year-old man diagnosed 'acute myocarditis': paired sera gave rising influenza C antibody titres of 32, 128 and high titres (256, 256) to *Mycoplasma pneumoniae*; Coxsackie B and other serological tests negative.

Case 4. Twenty-five-year-old man diagnosed 'acute pericarditis': paired sera gave falling CF antibody titres of 64, 16 with *M. pneumoniae* antigen; other tests not significant.

Case 5. Seventy-five-year-old man diagnosed 'myocardial infarction': paired sera gave rising CF antibody titres of 8, 32 with *M. pneumoniae* antigen; other tests not significant.

Case 6. Female infant diagnosed 'acute myocarditis': paired sera gave high

adenovirus group antibody titres of 256, 256, and adenovirus type 17 was isolated from stool; other complement fixation tests negative but rising titres (32, 128) of coxsackievirus B3 neutralizing antibody.

DISCUSSION

Virological tests can, with certainty or varying probability, demonstrate the presence of virus infection. They normally provide only partial and circumstantial evidence that the infection is responsible for associated illness, and absolute proof of aetiological relevance may be impossible in individual cases although it may be clearly demonstrable for groups of cases by epidemiological analysis. The high aetiological significance of the classical rising antibody titre (categories I and II of Table 1) arises from its demonstration of a close temporal relationship between the infective episode and the associated illness. Once elevated, coxsackievirus antibody titres decline variably over months or years, often with periodic anamnestic boosts from infections with other antigenically related enteroviruses, making interpretation of particular titres difficult: our data are presented in Table 1 in sufficient detail to allow readers to recalculate using different thresholds if desired. The serologically unsupported finding of an enterovirus in faeces provides unequivocal evidence of infection but does not distinguish coincidental, silent gut infection from an aetiologically significant condition. Because of these difficulties the present study is based on analysis of group data. Cases of serologically unsupported virus isolation (category VII) have not been counted as 'virologically positive' for the present analysis, though they were so included in our preliminary reports – they were few and their exclusion makes little difference to the outcome.

If a more rigorous threshold of positivity is applied to the analysis of data in Table 1, counting only categories I, II and III as positive, the percentages of cases above the threshold are 33 % for myocarditis (similar to 42 % for Bornholm disease, a known enteroviral disease which provides a 'positive control'), 13 % for pericarditis, and 4 % and 6 % respectively for 'other cardiac diseases' and non-cardiac diseases which provide almost 'negative controls'. Although the exact proportion cannot be defined, it is clear that Coxsackie B viruses make a major contribution to acute myocarditis and a significant one to acute pericarditis. This is true in each year of the study and in all age-groups (Tables 2 and 3). Cases occurred in all seasons of the year with lowest incidence in the first quarter and highest incidence of cases with rising antibody titres in the second and third quarters of the year corresponding to the seasonal distribution of enterovirus infections (Bell & Grist, 1973). Koontz & Ray (1971) reported similar findings from Seattle, with evidence of Coxsackie B virus infection in 44 % of myopericarditis cases and 11 % of non-cardiac illnesses but with no seasonal variation. Rising antibody titres were relatively less frequent in our cardiac cases than in Bornholm disease, suggesting that symptomatic cardiac involvement is a somewhat later complication of coxsackievirus infection than that of skeletal musculature.

Recent reviews by Lerner & Wilson (1973) and Abelmann (1973) show that while viral carditis is usually benign the illness may be serious and sometimes

severe, occasionally fatal and sometimes complicated by persisting myocardial inflammation, late sequelae including impaired heart function, E.C.G. abnormalities, and occasionally constrictive pericarditis. Speculation that coxsackievirus infection might initiate chronic cardiomyopathy and even valvular heart disease was strengthened by immunofluorescent demonstration of coxsackieviral antigens in human and experimental cardiac tissues (Burch *et al.* 1967; Burch *et al.* 1968; Burch & Colcolough, 1969). Our own serological studies did not suggest active or recent coxsackievirus infection in chronic cardiac diseases, nor did we find elevated Coxsackie antibody titres in five other cases of hypertrophic obstructive cardiomyopathy tested for Professor J. F. Goodwin. However, persisting high antibody titres would not be expected many years after an infection of average short duration if the chronic disease resulted not from persisting virus infection but from some other continuing process, possibly immunological, initiated by the virus infection.

Other viruses may also cause myopericarditis (Abelmann, 1973), including arboviruses which are common in many tropical areas where they may be important causes of acute, relapsing and chronic heart disease (Obeyesekere & Hermon, 1972). The serological results reported in our present paper suggest that, compared with Coxsackie viruses, the contributions of influenza, adenovirus, chlamydia, Q fever and *Mycoplasma pneumoniae* to myocarditis and pericarditis are small. One of the two patients with evidence of influenza (case 2) and the child with adenovirus type 17 infection (case 6) also showed serological evidence of coxsackievirus infection as an alternative cause of heart disease. Of the three patients with evidence of *M. pneumoniae* infection, one (case 4) also had serological evidence of influenza C, while in case 5 the illness was myocardial infarction. The lower frequency with which coxsackievirus infection was associated with pericarditis as compared with myocarditis in our series suggests that agents other than coxsackievirus might be implicated more often in pericarditis, though this was not supported by the relative frequency of infections diagnosed by complement fixation in myocarditis and pericarditis (Table 5).

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Post-transfusion hepatitis in a London hospital: results of a two-year prospective study

A Report to the M.R.C. Blood Transfusion Research Committee by the Medical Research Council Working Party on Post-Transfusion Hepatitis*

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SUMMARY

Seven hundred and sixty-eight patients were seen and tested at frequent intervals after transfusion of whole blood. Eight patients were judged to have developed icteric or anicteric post-transfusion viral hepatitis, an incidence of 1 %. Five were icteric and four of these were hepatitis B antigen (HB Ag) positive; two of these four died. One of the fatal cases and one non-fatal HB Ag positive case had received HB Ag positive blood. Two other antigen-positive patients had received blood or plasma or both which had not been tested for antigen.

Thirty-five patients showed conspicuous or sustained elevations of alanine transaminase without clinical features of hepatitis.

Four were positive for HB Ag but had not received antigen positive blood.

Two who had received antigen positive blood remained antigen negative, but one developed hepatitis B antibody (HB Ab).

Two other patients were also transfused with plasma.

Five had serological evidence of cytomegalovirus (CMV) infection accompanying the enzyme changes.

One patient who had received HB Ag positive blood remained antigen-negative and showed no abnormalities.

Five patients who became HB Ag positive, although they had been given antigen-negative blood, remained clinically and biochemically well.

Cytomegalovirus primary infection or reactivation occurred in another 32 patients; five had isolated, transient enzyme rises, one other was associated with a drug-induced focal liver necrosis and 26 showed no enzyme changes. Epstein–Barr virus infections, one of which was associated with a transient upset of enzyme activity, were detected in five patients. There were no cases of post-perfusion syndrome.

INTRODUCTION

Hepatitis of viral origin is a major public health problem throughout the world. It constitutes the main hazard of the transfusion of blood and the use of blood products apart from immunoglobulin and preparations of albumin. There are no reliable biochemical or histological features to help distinguish between type A (infectious) hepatitis and type B (serum) hepatitis. Both types of infection may be

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transmitted by blood transfusion and the term post-transfusion hepatitis includes both types. The clinical range includes inapparent infection, anicteric illness, acute icteric disease of varying severity and chronic liver damage.

A high incidence of post-transfusion icteric and anicteric hepatitis was reported between 1963 and 1970 in the United States of America, Germany and Japan (literature reviewed by Zuckerman, 1970*a* and 1972). No data for the incidence of icteric post-transfusion hepatitis in the United Kingdom more recent than 1954 are available and the frequency of anicteric post-transfusion hepatitis has not been previously investigated in this country apart from a small survey in Birmingham (Somayaji, Stone & Glover, 1967).

The discovery of a serum antigen associated with human hepatitis (Blumberg *et al.* 1967; Prince, 1968), now known as hepatitis B antigen (HB Ag), provided a serological marker for recognizing infection with or carriage of the virus of type B hepatitis. A test for HB Ag is now used to screen donor blood thereby reducing the incidence of transfusion hepatitis (McCollum & Zuckerman, 1970). Cytomegalovirus (CMV) and perhaps also Epstein–Barr virus (EBV) have recently been recognized as other possible causes of post-transfusion hepatitis (Paloheimo *et al.* 1968; Henle *et al.* 1970).

The two-year survey here reported was undertaken at the Central Middlesex Hospital, London, N.W.10, which serves a mixed population of about 250000 people. Approximately 4500 units of ACD blood, stored at 4° C., are used annually at this hospital, all of which are obtained from voluntary unpaid donors by the North London Blood Transfusion Centre, Edgware. Patients were studied intensively both clinically and by laboratory tests for liver function for 6 months after blood transfusion. The survey began on 1 July 1969 and the clinical follow-up of the last patient was completed on 31 December 1971. The objects of the survey were:

1. To obtain information about the incidence of icteric and anicteric post-transfusion hepatitis.

2. To establish the frequency of hepatitis B antigen (HB Ag) and the corresponding antibody (HB Ab) in blood donors and patients and to try to correlate their presence with blood transfusion and its complications.

3. To determine the frequency of transmission of EBV and CMV by blood transfusion and their role in causing post-transfusion liver damage.

METHODS

Patients and controls

All patients aged between 16 and 75 years who were transfused with whole blood at the Central Middlesex Hospital between 1 July 1969 and 30 June 1971, were invited to participate, after a full explanation of the purpose of the survey and the methods to be used. A pretransfusion blood sample was obtained for alanine transaminase (ALT) estimations and virological studies. Patients were seen at 2-weekly intervals for 3 months after transfusion and monthly for the next 3 months. Blood samples were obtained on each occasion.

At the initial interview a full history was obtained with particular reference to

previous jaundice, hepato-biliary disease and transfusion. Any history of injections, dental procedures, exposure to jaundiced persons and travel outside the United Kingdom within the 6 months before transfusion was noted. Details of alcohol consumption and all drugs being administered were noted; the illness necessitating hospital admission, and the nature of any anaesthetics given, were documented. Serial numbers of units of blood administered and where relevant those of other blood products were recorded. Defaulters who failed to respond to postal or telephone reminders were visited at home when possible.

At each follow-up visit patients were again questioned about these points and about possible symptoms of hepatitis. They were also clinically examined on each occasion. The serum ALT activity was measured at each visit. Patients with raised serum ALT values were recalled in order to confirm the abnormality, and measurements of serum bilirubin, van den Bergh reaction, alkaline phosphatase and bromsulphthalein (BSP) retention were carried out. If, as a result of these tests, hepatitis was suspected, haemoglobin, blood film, white blood cell count and differential, platelet count, prothrombin time and erythrocyte sedimentation rate were also studied. Patients with a confirmed serum ALT abnormality, together with either abnormal BSP retention or symptoms or signs suggesting hepatitis, were considered candidates for liver biopsy. Biopsy was never performed without informed consent by the patient. In addition the permission of the consultant in charge of the case, or of the general practitioner, was obtained. Biopsy specimens were processed for light microscopy by routine methods. Patients with confirmed hepatitis were seen frequently and treated according to conventional practice.

Initially, it was intended that the transfused patients would act as their own controls, since pre-transfusion biochemical and serological data were obtained from each patient. However, certain of the early results of tests for the presence of HB Ag and HB Ab indicated the need for untransfused controls. An attempt was made to follow a group of untransfused patients matched for age and sex with those transfused, at less frequent intervals for 6 months. After recruitment of 200 such controls this attempt was abandoned because it proved impossible to persuade the subjects to attend regularly for examination. Instead, untransfused patients aged between 16 and 75 years who had been in-patients during the preceding 6 months were randomly studied when they re-attended the out-patient department. A single blood specimen was obtained from each of them during the period 1 February to 30 November 1971. Although each patient gave only one sample, the total control group provides a range of specimens spanning a 6-month post-admission period. HB Ag and HB Ab tests were performed on all these specimens, but since paired sera were not available, CMV and EBV antibody studies were not done. It is appreciated that the design of the control series was a compromise.

Blood specimens

At each visit 20 ml. blood were collected in a sterile container and allowed to clot at room temperature. Specimens were centrifuged at 4° C. and the serum

distributed aseptically into sterile 3 ml. screw-capped vials. Serum for ALT estimation was stored for a maximum of 48 hr. at 4° C.; serum for virological studies was stored at −20° C.

Biochemical methods

Alanine transaminase was estimated by the 'optimal' method of Bergmeyer & Bernt (1963) with a Unicam SP 800 A recording spectrophotometer. The decrease in optical density at 340 nm. for 5 min. at 30° C. was recorded. The results were expressed as International Units (iu) per litre at 25° C., using a conversion factor of 0·723. Reproducibility of the test and activity of the reagents were determined daily with a commercially available control serum before and after every batch of tests (Chemonitor II, Dade Laboratories, Miami). The method is known to give values notably higher than those obtained by commonly used methods with sub-optimal substrate concentrations (Giusti, Ruggiero & Cacciatore, 1969). The enzyme values obtained in transfused patients had a log-normal distribution. Values greater than 30 i.u. were regarded as abnormal. For administrative reasons it was occasionally necessary to measure the serum ALT by an automated version of the colorimetric method of Reitman & Frankel (1957). Values greater than 40 Karmen units were regarded as abnormal.

Total serum bilirubin was measured by the Technicon Laboratory Auto-Analyzer method N12 (normal values < 0·8 mg./100 ml.).

Total serum alkaline phosphatase was measured by the method of Bowers & McComb (1966) using a Pye Unicam recording spectrophotometer at 30° C. (normal value < 110 i.u./l.).

Bromsulphthalein (BSP) retention was measured 45 min. after intravenous injection of 5 mg. BSP per kilogram of body weight (normal value < 5 % retention at 45 min.).

Virological studies

Tests for HB Ag and HB Ab. Immunodiffusion tests were carried out as described by Zuckerman & Taylor (1969) by the double diffusion micro-Ouchterlony technique as modified by Prince (1968). Agarose gel (0·9 %) was prepared in a buffer containing 0·1 M sodium chloride, 0·1 M tris (hydroxymethyl) amino-methanol (pH 7·6 at 25° C.), 0·001 M ethylenediamine-tetra-acetic acid, and 1 mg./ml. protamine sulphate. Merthiolate was added to the gel to give a final concentration of 1/5000. Tests were performed in 190 mm. Sterilin plastic Petri dishes containing 15 ml. of 0·9 % agarose gel. A pattern of six equivalent wells surrounding a central well was used. The wells were 2·5 mm. in diameter and 4 mm. apart. Test sera were added to the peripheral wells and allowed to diffuse for 20 min. before adding antiserum to the central well. The gel was kept in a moist chamber at room temperature and read daily for 7 days. The antiserum was human plasma containing HB Ab; it gave reactions of immunological identity with other laboratory reference antisera and was subsequently shown to possess the specificity *ad*. A known HB Ag positive control human plasma was included in every plate; recently, this was also shown to carry the determinant *ad*. Each positive precipitin reaction was checked

for immunological identity by immunodiffusion and confirmed by complement fixation (Taylor, 1970) and, sometimes, by electron microscopy (Zuckerman, 1970*b*). All the specimens are now being re-examined by radioimmunoassay and the results will be published in due course.

Serological tests for cytomegalovirus (CMV) and Epstein–Barr virus (EBV) antibodies

The first (pre-transfusion) and final (tenth) serum specimens from each patient were examined simultaneously for CMV and EBV antibodies. Whenever a test showed the appearance of antibodies in a previously sero-negative patient, or a fourfold increase in the titre of pre-existing antibody, the intermediate serum specimens from the patient were then examined together with the first and tenth specimens in a single test in order to determine the time of the antibody conversion or booster response. Sera were examined for cytomegalovirus complement-fixing antibodies by the micro-titre technique, using unheated, cell-associated antigens prepared from the AD 169 strain of virus (Stern & Elek, 1965). EBV antibodies were estimated by the indirect immunofluorescent technique, using EB3 Burkitt tumour cells (Epstein, Achong & Barr, 1964), which had been maintained on an arginine-deficient medium for 5 days before use, and fluorescein-conjugated anti-human IgG globulin (Wellcome Laboratories) (Henle & Henle, 1966; Henle, Henle & Diehl, 1968).

RESULTS

Between 1 July 1969 and 30 June 1971, 2184 patients were transfused. Of these, 489 were outside the accepted age limits and 443 were omitted for other adequate reasons (e.g. unavailable for follow-up). Thus, 1252 patients initially agreed to participate. Of these, 301 were lost to the survey: 109 died of causes other than hepatitis, 12 were too ill to continue to attend, 40 moved away, 67 could not be traced, and 73 refused to continue. There remained 951 patients who were followed for 6 months, but in 183 of these the follow-up was considered inadequate because intervals between successive visits exceeded 4 weeks on one or more occasions. The remaining 768 patients were studied in detail and completed 6482 of their expected 6912 visits. All the data reported below concern only this final group of 768 patients.

There were 474 women and 294 men. The average age of the women was 42 and of the men 54 years. There were 262 immigrants: West Indies, 125 (16 %); Ireland, 48 (6 %); India and Pakistan, 38 (5 %); other countries, 51 (7 %). There were 261 (34 %) general surgical cases, 177 (23 %) gynaecological, 147 (19 %) general medical, 99 (13 %) obstetric and 84 (11 %) orthopaedic. At the time of transfusion 80 % of the patients had undergone a surgical operation.

A transfusion was defined as any continuous infusion of blood; 84 % of the patients were thus transfused on one occasion only. The remainder were multiply transfused. Any blood subsequently administered later than 2 months after admission to the survey was ignored in calculating the average units received by each patient. The total number of units of blood transfused was 2824, an average of

Table 1. *Groups of hepatitis patients*

	Total no.	HB Ag positive	CMV antibody response
Group I. Hepatitis confirmed by liver biopsy	6	2	0
Group II. Typical clinical and biochemical features of hepatitis (including icterus); liver biopsy not done	2	2	0
Group III. Raised serum ALT without other clinical features of hepatitis			
(*a*) liver biopsy did not confirm hepatitis	6	2	0
(*b*) liver biopsy not done	29	2	4 primary 1 booster

3·7 units per patient. Forty-eight patients (6·3 %) also received plasma or other blood products, none of which had been tested for the presence of HB Ag: these patients were excluded from the calculation of the incidence of post-transfusion hepatitis.

The mean duration of stay in hospital was 26 days with a range of 2–212 days.

Hepatitis patients

One hundred and fifty-eight patients developed raised serum ALT values after transfusion. These patients were investigated for conditions other than viral hepatitis (e.g. drug-induced liver injury) that might have caused the ALT rises. It was arbitrarily decided that where such other potential causes existed, the patient would not be regarded as suffering from viral hepatitis. Hepatitis, either icteric or anicteric, was judged to be present in eight patients (1·0 %); sustained elevation of ALT without other clinical features of hepatitis was present in another 35 (4·6 %) patients. The results are summarized in Table 1.

Group I

This group comprised four males aged 27, 60, 65 and 69 years who received 4, 8, 17 and 6 units of blood respectively, and two females aged 39 and 47 years who received 2 and 4 units of blood respectively. All showed typical histological features of acute hepatitis. Two had received HB Ag positive blood and themselves became HB Ag positive. Both developed jaundice; one died, the other patient made a full recovery after an illness lasting 8 weeks. Four patients remained HB Ag negative; one developed jaundice and showed massive necrosis on biopsy and three were anicteric. The three anicteric patients recovered fully after ALT rises lasting 2 and 3 weeks and 6 months respectively. The patient with massive necrosis had persistent biochemical changes and hepatic abnormalities which were still demonstrable at 18 months, when biopsy showed inactive macronodular cirrhosis. The incubation periods to the first observed ALT rise were 2, 6, 7 (2 patients), 8 and 11 weeks respectively.

Group II

Both patients in this group showed clinical features consistent with viral hepatitis. A 66-year-old man became HB Ag positive 10 weeks after transfusion with six units of blood. One week later his ALT value was 2950 i.u./l. and he died within a few days with fulminating hepatic failure. Consent for post-mortem examination was not obtained. A 25-year-old man became HB Ag positive with an ALT value of 1400 i.u./l. 14 weeks after the transfusion with 19 units of blood and one unit of plasma. He was lethargic, anorexic and jaundiced but recovered fully after an illness lasting 6 weeks. Biopsy was not performed because of a history of bleeding after previous surgical procedures.

Group III

The 35 patients in this group showed conspicuous or sustained serum ALT rises, which in seven were greater than 100 i.u./l. In two of these seven and in three of the other patients, the serum bilirubin was between 1 and 2 mg./100 ml. However, none had symptoms or physical signs suggestive of viral hepatitis. The group consisted of 13 men aged between 23 and 68 (mean age 47 years) and 22 women aged between 22 and 67 years (mean age 43 years). These patients received between 1 and 21 units of blood (mean 3·9 units). Two of them also received one and 19 units of plasma respectively. Six patients underwent liver biopsy within 7 days of the ALT rise, but none had ALT values above 100 i.u./l., though all had abnormal BSP retention and one had a serum bilirubin of 2·0 mg./100 ml. None showed the histological features typical of acute viral hepatitis. Two of them were HB Ag positive, but in both the blood transfused was HB Ag negative, as it was in the other four patients who were biopsied; one of the latter had also received plasma.

The remaining 29 patients were unwilling to undergo liver biopsy. Two received HB Ag positive blood, but neither developed positive antigen tests: one had an ALT value of 260 i.u./l. 5 weeks after transfusion with raised serum bilirubin and alkaline phosphatase concentrations and marked BSP retention; the other had an ALT value of 47 i.u./l. 5 weeks after transfusion and subsequently became HB Ab positive. Two other patients became HB Ag positive with simultaneous ALT rises 7 and 24 weeks respectively after transfusion, but the blood transfused was HB Ag negative. Thus, in six of the 35 patients the presence of HB Ag in either the blood transfused or the recipient was associated with ALT rises in the recipient's serum; liver biopsies done in two of these recipients, however, failed to show hepatitis. One further patient in the non-biopsied group also received plasma but remained HB Ag negative. Among the remaining 24 patients were four in whom the ALT rises were associated with the appearance of CMV antibody and one in whom a CMV antibody booster response occurred. None of these five patients became HB Ag positive and the blood transfused was HB Ag negative. None showed clinical evidence of the post-perfusion syndrome, i.e. fever, lymphocytosis, lymphadenopathy and hepatosplenomegaly were not observed.

Other patients showing ALT rises

The residual 115 patients who showed ALT rises after transfusion were thought not to have viral hepatitis, although liver biopsies showed features akin to hepatitis in five of these. Halothane was accepted as the cause in these five cases; the onset of the hepatitis was 2 weeks after transfusion in four and 10 weeks after transfusion (but one week after a third exposure to Halothane) in the fifth. One case relapsed on re-exposure to Halothane. Liver biopsy showed focal liver-cell necrosis in a sixth patient who had received Halothane; this patient also showed a CMV antibody booster response. Halothane was also thought to be responsible for ALT rises in two further patients who were not biopsied.

Drugs or alcohol were accepted as the cause of ALT rises in nine patients. Liver biopsy showed evidence of recent liver-cell necrosis or cholestasis in one of these following oral contraceptive drugs. The biopsy of a second patient who had received chlordiazepoxide showed only siderosis and mild non-specific reactive changes. A third biopsy in an alcoholic patient showed fatty change.

Acceptable reasons for ALT rises were present in 27 patients. Liver biopsy showed abnormalities other than hepatitis in three (fatty change in one, non-specific inflammation in one, primary biliary cirrhosis in one). The primary clinical condition of the patient was felt to provide adequate reason for the ALT rises in ten patients with carcinomatosis, five with severe sepsis, four with pulmonary embolism, two with hepatic cirrhosis, one with acute pancreatitis, one with acute ulcerative colitis and one with gallstones.

Fifty patients showed ALT rises 2 weeks after transfusion. In many, the value had returned to normal a week later and in the remainder, with the exception of four, the value was normal within 2 weeks. In these four patients the test could not be repeated for 4 weeks; the values were then found to be normal. All but five of these patients had been recently operated upon and the ALT rises may have been the non-specific effect of the surgical procedure. However, a primary CMV antibody response occurred in one and booster responses in three of these surgical patients.

The remaining 21 ALT rises occurred at longer intervals after transfusion; these, too, had returned to normal again within one week. Biopsy was done after one such rise occurring 4 weeks after transfusion; the histology was normal. An increase in pre-existing CMV antibody titre followed an enzyme rise occurring 6 weeks after transfusion in another.

No clinical case of post-perfusion syndrome was seen.

Liver biopsies

Liver biopsy was performed in 25 patients. In six the lesions of typical acute hepatitis were found. In a further six, in whom hepatitis had been suspected clinically, convincing histological evidence was not found, though two of the patients were HB Ag positive; any histological changes present in these six cases were very slight, ranging from mild sinusoidal congestion to occasional focal liver-cell necrosis. The biopsy appearances in 12 patients, whose raised ALT values were thought to

have causes other than viral hepatitis have already been mentioned. Biopsy in the remaining patient after an isolated ALT rise was normal.

HB Ag and HB Ab results

Recipients of positive donor blood

Of 8953 units of blood for transfusion, HB Ag was detected in 13 (0·15 %), and HB Ab in 8 (0·09 %): 2824 of these were transfused. Five patients in the survey received blood which was subsequently shown to contain HB Ag. Two developed icteric hepatitis with antigenaemia and one of them died. Another developed a persistently raised ALT but antigen was not detected in his serum, and in a further patient HB Ab appeared after a minor ALT rise; neither of these patients underwent biopsy. The fifth recipient had an entirely uneventful clinical course and tests for antigen and antibody were both negative. Two patients who received known HB Ab positive blood had an uneventful clinical course.

Prevalence of HB Ag and HB Ab in survey patients

One patient out of the 768 surveyed was HB Ag positive before transfusion and remained a persistent carrier; his liver function tests remained normal.

The antigen became detectable in the blood of 13 other patients, including the two mentioned above who received HB Ag positive blood. Icteric hepatitis occurred in four, two of whom died. Four others showed ALT rises; the remaining five patients had uneventful clinical courses. HB Ag was first detected in the serum of these patients at the following intervals after transfusion: after 7 and 10 weeks respectively in the two patients who died; after 4 and 14 weeks respectively in the two icteric patients who recovered; and 2, 4, 7 and 24 weeks respectively in the four patients who showed only raised ALT values. The five patients who had an uneventful clinical course showed antigenaemia 2 weeks (three patients) and 10 weeks (two patients) after transfusion.

HB Ab became detectable in the serum of seven patients (0·9 %). In one, who had received antigen-positive blood, the appearance of antibody coincided with an ALT rise 5 weeks after transfusion. This patient has been described under Group III above. In five who had received blood which was antigen-negative by the immunodiffusion technique antibody appeared 2, 14, 15, 19 and 20 weeks respectively after transfusion. The seventh patient received blood which had not been tested for the presence of antigen, and antibody was first detected 4 weeks after the transfusion. These six patients showed no rise in ALT values.

Three patients, who had detectable HB Ab before transfusion, remained persistent antibody carriers throughout the survey. One of these showed a raised ALT 2 weeks after blood transfusion, possibly attributable to three successive exposures to Halothane. The other two carriers had normal liver function tests. None of these three patients had a past history of transfusion or hepatitis.

Control patients

Between 1 February and 30 November 1971, 1489 patients agreed to act as controls. There were 716 men (average age 53 years) and 773 women (average

Table 2. *Summary of HB Ag and HB Ab results*

	No. tested	HB Ag positive	HB Ab positive
Transfused patients	768	13 (1·69)	7 (0·91)
Blood donors	8953	13 (0·15)	8 (0·09)
Control patients	1489	5 (0·34)	7 (0·47)

Figures in parentheses indicate percentages.

age 48 years). They comprised 606 general medical, 181 general surgical, 457 orthopaedic and 245 obstetrical and gynaecological patients. Raised ALT values were found in 42. Adequate explanations for these, other than hepatitis, such as congestive heart failure, were present in 20. In another five patients ALT returned to normal within one week. In a further six, liver biopsies did not confirm a diagnosis of hepatitis. The remaining 11 cases possibly did have hepatitis; one was confirmed by liver biopsy, three others became HB Ag positive, and one became HB Ab positive.

HB Ag was found in five control patients (0·3 %) including the three with ALT rises mentioned above; the other two appeared to be symptomless carriers.

HB Ab was found in seven control patients. One initially had a raised ALT (see above) which may have been due to hepatitis. Another had a long standing hepatosplenomegaly of unknown cause with normal liver function tests, and the remaining five were clinically normal.

The findings with respect to HB Ag and HB Ab in transfused patients, blood donors and control patients are summarized in Table 2. The difference in the frequency of antigen in the transfused patients on the one hand and the control patients and blood donors on the other is significant at the 1 % level. The difference in frequency of antigen between the control patients and blood donors is not significant. There is also a statistically significant difference in the frequency of antibody between transfused patients and the blood donor population, but not between transfused and control patients.

Cytomegalovirus and Epstein–Barr virus infections

Serial serum specimens were available for testing in 712 of the 768 patients: there were 270 men and 442 women.

CMV primary infections: 270 patients (38 %) had no detectable CMV antibody before transfusion. Twenty-four of these (9 %) acquired antibody after transfusion. Thirteen were women, mean age 39 years, and eleven were men, mean age 57 years. They had received between one and 14 units of blood (mean 4·4 units). Antibody appeared 4 weeks after transfusion in 15 patients, after 6 weeks in two, after 10 weeks in four, and after 12 and 24 weeks respectively in two. The time of conversion is not known for one patient whose intermediate sera were not tested. A sustained ALT rise immediately preceded the antibody conversion in three patients and followed it within an interval of 6 weeks in one but the patients showed no other features of hepatitis (in Group III above). Another patient showed a

transient ALT rise 2 weeks after transfusion but the antibody rise in this patient did not occur until at least 12 weeks later. In one patient the antibody appeared after an influenza-like illness during which liver function tests remained normal. The remaining 18 conversions occurred without symptoms or biochemical abnormalities.

CMV antibody booster responses: 442 patients (62 %) had CMV antibodies at the time of transfusion. Thirteen of these (2·9 %) showed a fourfold increase in antibody titre. There were nine women, mean age 43 years, and four men, mean age 62 years. They had received between one and 12 units of blood (mean 3·6 units). Antibody titres rose after intervals from transfusion of 4 weeks (three patients), 6 weeks (two patients), 8 weeks (two patients) and 10 weeks (four patients). In two, the exact time of the rise in titre was not determined. These booster responses occurred predominantly in patients with low initial antibody titres; they were not found in patients with initial titres of 1/128 or more. One of these patients developed an ALT value of 36 i.u./l. 6 weeks after transfusion and the changes persisted for 4 weeks (Group III above). Four others showed transient ALT rises, 2 weeks after transfusion in three patients, and 6 weeks after in one; the antibody booster responses in these four patients occurred 2, 4, 6 and 6 weeks after the ALT rise. One further patient had shown conspicuous elevations of ALT before a rise in CMV antibody titre, but these were thought to have been caused by Halothane; biopsy revealed a focus of liver-cell necrosis. The remaining seven patients with booster responses had neither symptoms nor biochemical abnormalities.

Persistent abnormalities of liver function, without other evident cause, were thus demonstrated in five of the 37 patients undergoing either primary CMV infection or CMV reinfection; five showed transient ALT abnormalities.

EBV primary infection: 55 patients (7·7 %) had no EBV antibody before transfusion. Three of these (5·5 %) developed antibody after intervals from transfusion of 2, 2 and 24 weeks respectively. No disturbance of liver function or clinical illness developed in these patients. A fourth patient, who showed an EBV antibody conversion after 10 weeks, had intermittent ALT rises throughout the survey; these might have been caused by alcohol or treatment with antidepressant drugs.

EBV booster responses: 657 patients had EBV antibody before transfusion. Only one of these showed a fourfold increase in titre 4 to 6 weeks after a transient rise which occurred 4 weeks after transfusion.

Thus there was serological evidence of EBV infection in five patients.

DISCUSSION

The overall incidence of icteric and anicteric hepatitis in the present survey (1·0 %) is low compared with the incidence found in prospective studies in Japan (65 %, Shimizu & Kitamoto, 1963), U.S.A. (18 %, Hampers, Prager & Senior, 1964), and Germany (14 %, Creutzfeldt *et al.* 1966). It is important to consider whether this difference is real, resulting, for example, from a lower incidence of carriers of hepatitis B virus in the donor population, or apparent because of the

differences in the patient populations studied or in the criteria used in diagnosing hepatitis.

The only exclusion of patients in the present survey was made on the basis of extremes of age or unwillingness to co-operate. With regard to the latter no important differences in terms of age, sex, primary diagnosis, country of origin, previous liver disease, recent contact with jaundiced persons, injections received outside the hospital, recent travel abroad or the survey month in which they were transfused were found between patients in the trial group and those who were omitted. Nor was there any significant difference in the volume of blood received by these two groups. There is therefore no reason for assuming that the omitted group might have been at greater risk of developing hepatitis. Overcrowding and poor social conditions are not uncommon in the background of the patients studied. Cherubin (1971) has drawn attention to the low incidence of hepatitis in a Harlem hospital despite the use of commercial blood. He attributed this to immunity resulting from exposure to hepatitis B virus in a lower socio-economic urban community.

Criteria used for the diagnosis of hepatitis are next considered. Liver biopsy may provide incontrovertible confirmatory evidence of hepatitis but this procedure is seldom undertaken. When liver biopsy was not performed, that is to say in the majority of the survey patients, reliance was placed on clinical evidence and measurement of serum ALT. The duration and degree of elevation of the enzyme that qualify a patient for inclusion in the hepatitis group must be critically examined. Neither a rise in ALT, nor its magnitude, is a specific indication of hepatitis. In some previous surveys a transaminase level was arbitrarily defined below which a diagnosis of hepatitis was not made. Shimizu & Kitamoto (1963) required the ALT to reach a value greater than 90 units (normal < 40 units) and to remain above 50 units for at least 2 weeks. Hampers, Prager & Senior (1964) required elevations of at least three standard deviations above the mean on at least two occasions. Katz, Rodriguez & Ward (1971) in Chile adopted an ALT > 100 units for more than 10 days as indicating hepatitis. In the present survey no such arbitrary lower limit was set. However, if other factors were present which might have caused the enzyme rise these patients were not considered to be suffering from viral hepatitis; it was accepted that these other factors were a more likely cause of the liver damage. These included such important categories as underlying hepatobiliary disease and the administration of potentially hepatotoxic drugs. This rigid exclusion of all patients having other possible causes for their liver damage may have contributed to the low incidence of hepatitis in the present study.

Another factor which must be considered in this context is the fact that only 25 (29 %) of the 87 patients with sustained ALT rises underwent liver biopsy and this is the conclusive way in which hepatitis can be proved. In the absence of histological proof, the significance of conversion to HB Ag positivity in conjunction with an ALT rise was regarded as evidence of viral liver damage only in two patients who developed a frank clinical illness. Without the latter the relationship is more speculative and histological studies in two such patients failed to reveal any hepatitis. These cases were therefore also excluded from the hepatitis group.

Similarly, ALT rises alone were not regarded as providing sufficient evidence of hepatitis.

The techniques used during the survey for detecting HB Ag and HB Ab were not as sensitive as those now available, e.g. radioimmunoassay, and therefore the figures now reported probably underestimate the frequency of antigen and antibody. The results of examination by radioimmunoassay of the specimens collected in the survey, all of which were stored frozen, will be published later.

A further possible cause of underestimation of the incidence of post-transfusion hepatitis must lastly be considered. During the last five months of the survey the blood used for transfusion had been screened for HB Ag by the immunodiffusion technique and 23 % of the patients received such blood. Of the eight patients with hepatitis five, including two who became antigen positive and one of whom died, developed hepatitis after the screening programme began at the Regional Transfusion Centre. The risks still attached to transfusing screened blood have been stressed by Alter *et al.* (1972). The sensitivity of screening tests and the possible role played by different subtypes of hepatitis B virus in causing liver damage attributable to blood transfusion may be important.

Somayaji, Stone & Glover (1967) reported a prospective study in which 45 patients were surveyed, including measurement of serum transaminases, for 22 weeks after transfusion. Only two showed a transient elevation of serum enzymes; liver biopsy on one of these showed normal liver tissue. The authors concluded that none of the 45 patients developed hepatitis. The main evidence for the incidence of post-transfusion hepatitis after blood transfusion in the U.K. is derived from three prospective studies, in which the occurrence of jaundice was the criterion by which the diagnosis of hepatitis was made; Spurling, Shone & Vaughan (1946) found six (0·7 %) cases of jaundice among 891 patients but these were considered 'doubtful if due to transfusion'; Lehane, Kwantes, Upward & Thomson (1949) observed 22 cases (0·8 %) among 2796 patients; in a joint survey organized on behalf of Ministry of Health, Medical Research Council and Department of Health for Scotland (Ministry of Health, 1954), there were four cases (0·16 %) of jaundice among 2538 patients in England and Wales and five (0·36 %) among 1387 patients in Scotland.

The low incidence of hepatitis in the present study must also be considered in the light of transfusion procedures in different countries. Evidence from the United States indicated that blood obtained from paid donors carried a greater risk of transmitting hepatitis in the recipient than blood from unpaid volunteers (Allen *et al.* 1959; Grady & Chalmers, 1964; Walsh *et al.* 1970). In the present study blood from unpaid donors was exclusively used. Nevertheless, although the incidence of hepatitis was small there was a morbidity and mortality equivalent to 27 cases of hepatitis, including eight deaths, per 10,000 units of blood transfused in patients receiving blood only.

The frequency of CMV and EBV antibodies in the hospital population studied here was similar to that found previously for the same age groups in London (Stern & Elek, 1965; Pereira, Blake & Macrae, 1969). Although the risk of infection with these viruses, after blood transfusion, is greater with fresh blood (Paloheimo *et al.*

1968) the present investigation confirms that infection can be transmitted by blood stored for several days (Stevens, Barker, Ketcham & Meyer, 1970; Prince, Szmuness, Millian & David, 1971). Although most infections are primary ones in patients without antibody, reinfections or reactivations of latent infections do occur, particularly in patients with low titres of pre-existing antibody. Nearly all of these infections are symptomless.

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The use of inactivators in the evaluation of disinfectants

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SUMMARY

Since the earliest days of disinfectant testing bacteriostatic effect has misled many workers in this field. This problem has long been appreciated by some and a considerable battery of neutralizers has been employed, often with great success. Other anti-bacteriostatic measures, used without control, fail to revive damaged though viable organisms.

This paper sets out to describe some of the problems that are encountered in this confused field and the means whereby they may be overcome.

INTRODUCTION

Many pitfalls beset the complex and much fought over subject of chemical disinfectant testing. Not the least of these is the omission, incorrect choice or misuse of an inactivator to neutralize bacteriostatic effect. Much of the basic information on this subject has been available for a long time but it has remained unappreciated by some workers. It therefore seems timely to draw attention, once again, to these matters.

Extravagant claims have been made and much misplaced enthusiasm shown for the disinfectant virtues of particular products when the unwary have mistaken bacteriostasis for a true kill. The error may be exaggerated when an incorrect or insufficient inactivator is employed. Too powerful an inactivator may complete the work the disinfectant has often failed to do.

Misconceptions linger to this day, often influenced by earlier and equally misconceived reports. Many publications on disinfectant testing still fail to recognize the misleading effect of inhibition by the disinfectant or by the so-called inactivator. However there is a growing appreciation of the need for controlled inactivation.

The viability of an organism is most commonly demonstrated by its ability to reproduce. For nearly a century, however, it has been recognized that organisms which have been treated with a disinfectant and which fail to grow *in vitro* and are thus presumed dead, may, in fact, still retain their pathogenicity (Geppert, 1889).

THE PROBLEMS

Bacteriostasis will arise as a result of disinfectant carried over into the recovery medium. It will also occur when disinfectant molecules become attached to the

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cell wall of the organism by weak chemical bonds. Washing the organism, or voluminous dilution, with a non-inactivating medium may overcome the former but is quite ineffective against the latter (Chiori, Hambleton & Rigby, 1965).

The realization that many types of disinfectant are bacteriostatic at vastly greater dilution than those at which they are quickly bactericidal has led to the use of a wide range of chemical and, occasionally, physical neutralizers. The choice of an inactivator should be strictly limited by certain criteria. It must, by definition, neutralize the disinfectant it is used against. It should not give rise to any inhibiting effect, either of its own or as a result of any products formed when it is combined with the disinfectant. Its action should also be fairly rapid; slow neutralization allows continued bactericidal effect long after any timed period has ended.

In reality few, if any, of the commonly used inactivators are completely non-inhibitory and great care must be taken in the use of higher concentrations. Inactivators are employed either as an intermediate stage for diluting or neutralizing any disinfectant carried over before transfer to a growth medium or they may be included in the growth medium itself, be it liquid or solid. Both systems have their disadvantages. In the first, small numbers of survivors may become less apparent simply through dilution. In the second, the inactivator's own inhibitory power tends to have greater effect.

Checking the performance and suitability of an inactivator is no easy process.

First, it must be appreciated that organisms that have been physically or chemically damaged may require growth conditions very different from those needed by undamaged organisms. Low concentrations of disinfectants have been employed to bring about the mutation of organisms, resulting in much altered nutritional requirements (Englesberg, 1952). Selection of a suitable medium for recovery can be done only on the evidence of damaged organisms, a point strongly emphasized by Harris (1963). As a result of damage to the organism the inactivator may exert a different effect (Valko & Dubois, 1944).

No hard and fast rules can be laid down regarding inactivators – the great variety of organisms and test methods prevents this; but general guidelines, before specific checking, may be of some value.

No individual test can supply all the information regarding the inactivator's performance. A modification of the British Standard 3286: 1960 method of checking inactivators may act as a guide. But when recovering damaged organisms only the quantitative comparison of a variety of inactivators and conditions of use will lead to the most advantageous choice at that time.

It will be seen from a study of published work that this procedure is not normally carried out. Strong evidence, especially from non-commercial bodies, is now available to demonstrate the necessity of checking inactivators before and during use. The blind assumption that any concentration of inactivator will work all the time is not good enough.

INACTIVATORS IN COMMON USE

Many different inactivators are in vogue, most often, it seems, as a result of tradition rather than proved necessity. Detailed reading will bring to light countless others that have been superseded by more 'impressive' products. Those in current use include the thioglycollate, thiosulphate and bisulphite salts of sodium, the surfactants Lubrol W, Tween 80 and Lissapol N and various organic compounds of animal origin such as lecithin, cholesterol and serum.

Sodium thioglycollate

Geppert used ammonium disulphide as an inactivator for mercuric chloride. It was later found that the thiols, usually in the form of sodium thioglycollate, would perform the same function (Marshall, Gunnison & Luxen, 1940). So apparently successful was thioglycollate broth, both as an inactivator and in its designed purpose as an anaerobic medium (Brewer, 1940), that its use for sterility testing was made official (U.S.P. XVI 1960). The wisdom of this is to be doubted. Examination of work by McClung (1940) shows that for small numbers of organisms – a condition likely to be met in disinfectant testing – recovery was better in media containing no thioglycollate. Engley & Dey (1970) have reported a toxic effect of thioglycollate against, in particular, *Staphylococcus aureus*. A similar result was obtained in this laboratory against *Pseudomonas aeruginosa* and other organisms.

The status of thioglycollate broth as the official medium for sterility testing in the United States has led to its misuse as a recovery agent against such diverse products as buffered glutaraldehyde and chlorine dioxide (commercial literature). Though undoubtedly effective in neutralizing mercurial compounds, its toxic nature must give rise to concern.

Sodium thiosulphate

Another much misused chemical is sodium thiosulphate. It was employed initially, at very low concentrations (0·1 ml. of 3 % solution per 170 ml. sample) to neutralize chlorine residues in water samples (Mallman & Cary, 1933). For this application the results showed the need for such inclusion. A slightly higher concentration of 0·5 ml. of 1 % solution, per agar plate, was used by Mudge & Smith (1935). Their results showed that, even at this still comparatively low concentration, in 6/18 samples growth was seriously diminished by the presence of the thiosulphate. In a further 2/18 samples growth was not improved by its use.

It can be seen that even this small amount of thiosulphate, when included in the growth medium, has a profound effect on the growth of survivors. Concentrations varying between 0·1 % and 5 % have been used, mostly without any apparent form of control.

Reports on the toxic nature of sodium thiosulphate against, in particular, staphylococci (Kayser & van der Ploeg, 1965; Gross, Cofone & Huff, 1973) agree with unpublished results from this laboratory. The degree of inhibition will vary in different basal media. Other damaged organisms which have shown themselves to be affected include the more chemically resistant pseudomonads and mycobacteria.

Thiosulphate has also proved popular for use against the iodophors. Unfortunately sodium thiosulphate reacts with iodine to form sodium tetrathionate. This compound is itself an inhibitor and as such is used as a selective agent for the isolation of salmonellas.

Elemental iodine is sufficiently inactivated by the organic material in nutrient media (Gershenfeld & Witlin, 1950) as are chlorine and hypochlorite. This makes the use of sodium thiosulphate unnecessary. The inclusion of more organic material in the form of serum may be of some value where higher concentrations of these disinfectant ions are countered.

Sodium bisulphite

Sodium bisulphite is recommended as an inactivator for glutaraldehyde and occasionally against formaldehyde (Rubbo & Gardner, 1965). Though it is often included in growth media, it should only be used as an intermediate diluting stage.

Engley & Dey (1970) have reported the inhibitory nature of bisulphite at low concentrations. Bergen & Lystad (1972) found it a better bactericide than glutaraldehyde alone.

Sodium bisulphite is not used in this laboratory. Nutrient broth has given the best recovery results, provided the concentration of aldehyde in the medium is kept sufficiently low.

Further study possibly based on the work of Nash & Hirsch (1954), may eventually provide a more effective inactivator.

Surfactants

Ever since the introduction of the quaternary ammonium compounds, diguanides and ampholytes as disinfectants, they have been recognized as being bacteriostatic at very high dilutions. Normal or enriched recovery media, though partially effective in reducing the activity of these compounds, are insufficient as inactivators.

Various detergent surfactants have been quoted as enhancing the recovery of inhibited organisms.

Anionic and cationic surfactants often prove themselves very effective inactivators when used in conjunction with undamaged organisms. However they are toxic (Weber & Black, 1948) and should not be used for the recovery of damaged organisms, especially those that are Gram positive.

Nonionic surfactants such as Lubrol W, Tween 80 and Lissapol N have only low toxicity levels against most organisms, with the result that high concentrations may be used, e.g. 7 % Lubrol W (Bergen & Lystad, 1972).

Tests performed in this laboratory show that all three compounds will work well in a given situation. Variations of the disinfectant or the test organism may require the use of a different inactivator. For example, Lubrol W at concentrations of 0·1 % and above is reported as being inhibitory to streptococci. Tween 80 should be used instead (Imperial Chemical Industries, 1973).

Phenol and the phenolics require inactivation (Flett, Haring, Guiteras & Shapiro, 1945) for which Tween 80 appears to be suitable (J. F. Gardner, personal communication).

Table 1

Disinfectant	Inactivator
Aldehydes	Enriched nutrient medium or see Nash & Hirch (1954)
Phenolics Pine Fluids	A non-ionic surfactant, Tween 80 should be the first choice
Ampholytes Diguanides Quaternary ammonium compounds	A non-ionic surfactant, possibly with lecithin
Mercurials	Sodium thioglycollate at a very low concentration
Elemental iodine Bromine Chlorine Hypochlorites Hypobromites	Nutrient medium possibly with sodium thiosulphate at a low concentration
Iodophors Hypochlorites + detergents Hypobromites + detergents	A non-ionic surfactant, Tween 80 should be the first choice

Modern iodophor preparations require inactivation not, as is commonly assumed, by sodium thiosulphate but by a nonionic surfactant. Again Tween 80 produces much improved recovery results.

Surfactants cause lysis of blood. Lower colony counts may occur when these compounds are used as diluents when transfer is to blood agar plates (Davies, 1949). Another form of enriched agar should be used in such cases.

Animal products

Lecithin, cholesterol and serum have been variously described as inactivators. Lecithin is popularly employed with Lubrol W or Tween 80 (Letheen broth).

The use of cholesterol was advocated by McCulloch, Hauge & Migaki (1949) as it was an important constituent of brain and egg yolk, both of which had proved effective inactivators for quaternary ammonium compounds. Its insolubility makes it difficult to use and, as with lecithin, recovery results are very variable.

Serum has little value as an inactivator except against halide ions. It is, on the other hand, of undoubted value as an enrichment for damaged organisms, a subject not covered by this paper.

SUGGESTIONS FOR IMPROVED INACTIVATION

A list of potential inactivators is shown in Table 1. A suggested method of checking them may be of some assistance.

The initial selection of any inactivator may be made using a modification of the British Standard 3286: 1960 method. The modification consists of incubating the containers of inactivator and of inactivator plus disinfectant when the normal test is completed. Growth should occur in both containers. As this test is performed with undamaged organisms it is an insufficient guide on its own.

Final selection of the inactivator and the concentration to be used is probably best made by the following procedure.

1. The organism is subjected to the normal disinfectant test.
2. Similar samples of the disinfectant/organism mixture are placed in a variety of possible inactivators and incubated.
3. The inactivator which allows growth in the greatest number of samples, or, which gives the most prolific growth should be considered the most suitable.
4. Once a particular inactivator has been selected the test should be repeated using a range of concentration of that inactivator.

This procedure must be repeated whenever any variation, such as a change of organism, is introduced into the test.

The same principle may be applied in the selection of the recovery medium.

In every case the concentration of disinfectant in the recovery medium should be kept very low.

Inactivators may be included either in solid or liquid media. Recovery results are often much poorer on agar. Unpublished work in this laboratory shows that wherever possible a liquid inactivator medium should be used.

Damaged organisms invariably have an extended lag phase. Incubation periods of seven days or more should be considered normal. The commonly accepted 48 hr. incubation period is often insufficient.

CONCLUSION

The need for an inactivator step in disinfectant testing must be quite obvious. The necessity of controlling this step is less well appreciated.

Variations between organisms, the damaging agent and the degree of damage will all serve to alter the recovery requirements.

Most commonly used inactivators are inhibitory to some damaged organisms though this may not be apparent from experimental results based on undamaged organisms. Only by the comparison of a variety of inactivator media and conditions, while recovering damaged organisms, will a more suitable choice be made.

The ultimate truth regarding the best recovery conditions may never be known though every effort should be made to enhance the growth of the damaged organisms.

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Oral dose and faecal concentration of antibiotics during antibiotic decontamination in mice and in a patient

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SUMMARY

In both mice and one patient, a similar correlation was found between the oral dose of a 'nonabsorbable' aminoglycoside antibiotic selected for antibiotic decontamination of the digestive tract and the resultant faecal concentration. In mice, absorption of the orally-given antibiotics could only be demonstrated in animals treated with extremely high doses of 1440 mg. per kg. body weight per day. Evidence of absorption of gentamycin was found to occur in a patient after doses as low as 70 mg. per kg. per day.

INTRODUCTION

In man as well as in animals bacterial infections during and as a consequence of a severe decrease in defence capacity are more often prevented by previous antibiotic decontamination of the digestive tract. The latter is decontaminated because it appears to be the main source of bacterial invasion in such individuals. The antibiotics used for decontamination are administered orally and are not or only minimally absorbed from the digestive tract. The antibiotics employed for decontamination in man are sometimes selected on the basis of a sensitivity test (Gunn & Gould, 1965) but are often selected without prior information concerning the sensitivity pattern of the patient's microflora and are supplied in empirical dosages (Schwartz & Perry, 1966; Bodey, Loftis & Bowen, 1968; Preisler, Goldstein & Henderson, 1970). In our laboratory, decontamination of animals is always preceded by a sensitivity test on their faecal microflora (van de Waaij, de Vries & Lekkerkerk, 1970). In this way, an optimum mixture of antibiotics is selected in a first phase of the sensitivity test. In a second phase, the minimum bactericidal concentration of the selected antibiotics for the intestinal flora to be eliminated is determined. It is still a matter of trial and error to determine at which dose the antibiotics must be supplied in order to achieve at least the minimum bactericidal concentration in the intestines. Therefore, the relation between various oral dosages of neomycin and the resulting faecal concentration was investigated in mice while similar observations were made in a patient treated with gentamycin. We only once had a patient with an extremely resistant microflora so that oral treatment had to be started with quite high doses. Dose reduction could for the same reason

only be performed stepwise so that the concentrations resulting from three doses could be studied.

The relation between the faecal concentration and the concentration of the antibiotics in the serum was also determined both in the patient and in mice.

MATERIAL AND METHODS

Mice

The animals were conventional female ND2 mice of 12 weeks of age with an average body weight of 35 grams. They were housed in separate cages with individual drinking water bottles for the estimation of the daily water (antibiotic) intake. Autoclaved pelleted food (Hope Farms) was supplied *ad libitum*. The animals were maintained under strict isolation conditions in autoclaved cages inside a 2% peracetic acid-sterilized laminar cross-flow bench (van der Waaij & Andreas, 1971).

Bacteriological culturing

Fresh faeces were taken daily for culturing in Brain Heart Infusion broth and in Brewer's semi-solid thioglycolate medium at 37° C. The cultures were incubated for 1 week before they were determined negative when no evidence of growth was seen. If growth was observed, the cultures were subcultured for pure culturing and subsequent identification.

Patient

A 12-year-old boy, weight 30 kg., was decontaminated and maintained in a down-flow isolator (van der Waaij, Vossen & Korthals Altes, 1973) under strict bacteriological isolation conditions. The child was suffering from severe Pemphigus vulgaris and frequent periods of bacteraemia due to *Staphylococcus aureus*. This strain was resistant to all antibiotics except gentamycin after 1½ years in hospital, which justified our unconventional approach to treatment. To minimize bacterial infection of the skin lesions, antibiotic decontamination was done as well as skin disinfection by twice daily bathing in 0·5% solution of chlorhexidine in water. Faeces and blood were sampled daily for culturing and the estimation of the antibiotic concentration. Blood was also investigated biochemically to monitor liver and kidney function. The biochemical tests involved were: serum creatinin concentration, and the S.G.O.T., S.G.P.T. and alkaline phosphatase tests.

Antibiotic treatment and sampling

On the basis of a sensitivity test (van der Waaij *et al.* 1970), a combination of neomycin and bacitracin was found to be most suitable for decontamination of the mice. According to the results of a sensitivity test of the same type, the patient was treated with a combination of gentamycin and bacitracin. The mice were treated in groups of 28 animals; each group received 1·25, 2·5, 5·0, or 10·0 mg. of neomycin per ml. of water in combination with 2·5 mg. bacitracin and 100 μg. pimaricin per ml. According to the daily water intake this equalled, respectively, 180, 360, 720, and 1440 mg. neomycin/kg. body weight per day.

Immediately after daily sampling in eight mice per dose, the faeces were suspended 1/10 in tryptose phosphate (T.P.) broth (DIFCO) for the determination of the neomycin concentration. The animals were killed in groups of four on days 2, 3, 4, 5, 7 and 12 of treatment. For the determination of the antibiotic concentrations in the serum, blood was sampled immediately after killing.

The patient was given 2 g. of gentamycin every 6 hr. (about 280 mg./kg./day) for 4 successive days. Thereafter, the dose was reduced to 1 g. four times a day (about 140 mg./kg./day) again for 4 days. Treatment of the patient was then continued with 0·5 g. gentamycin 4 times a day (about 70 mg./kg./day) for 15 days. Bacitracin was given in combination with gentamycin from the beginning of treatment in a dose of 1 g. and nystatin in a dose of 500,000 i.u. during the first 8 days; during the second treatment period of 15 days, these doses were, respectively, 0·5 g. and 500,000 i.u. administered four times a day.

Faecal samples from the patient were also suspended 1/10 in T.P.-broth for the determination of the gentamycin concentration.

Antibiotic concentration assay

The concentration of neomycin and gentamycin in the faeces and the serum was determined by the microcup dilution method described by Goss & Cimyotti (1968). This test gave good reproducible results after it had been standardized and reference curves were determined (W. D. H. Hendriks *et al.*, to be published). The test was performed with a strain of *Escherichia coli* which was sensitive to 1 μg./ml. gentamycin and to 3 μg./ml. neomycin. A concentration of 10^4 *E. coli* cells per ml. of T.P.-broth, with tetrazolium tetrachloride (T.T.C.) as indicator, was used for the test. This assay was not applicable to bacitracin, since we failed to isolate a streptococcus strain with an adequate sensitivity for bacitracin which reduced the medium sufficiently for a change in the T.T.C. Other methods for determining the bacitracin concentration in the faeces also failed to give sufficient accuracy.

RESULTS

Mice

The relation between the oral dose of neomycin, and the faecal concentration is presented in Figs. 1 and 2. It took 7–10 days before the final concentration was reached in the colon as evidenced by the concentration in the faeces (Fig. 1).

The oral intake of the antibiotic mixtures was in the normal range of 4–5 ml. per day after it had been lower in the first 3–4 days of treatment in the mice treated with neomycin doses up to 720 mg./kg./day. The dose of 1440 mg./kg./day gave more difficulties in this respect. Some animals refused to take it in sufficient amounts for several days, so that they had to be replaced by others.

Apart from the mice treated with 170 mg. neomycin the faecal cultures remained sterile from the second day. However, the majority of the mice had sterile faeces from the third day. The rest of the animals gave sterile cultures on day four. It was also interesting that the higher doses of 720 and 1440 mg. per kg. body weight per day resulted in progressively higher faecal concentrations than did the lower doses (Fig. 1). In the group treated with 180 mg./kg./day, two animals that were

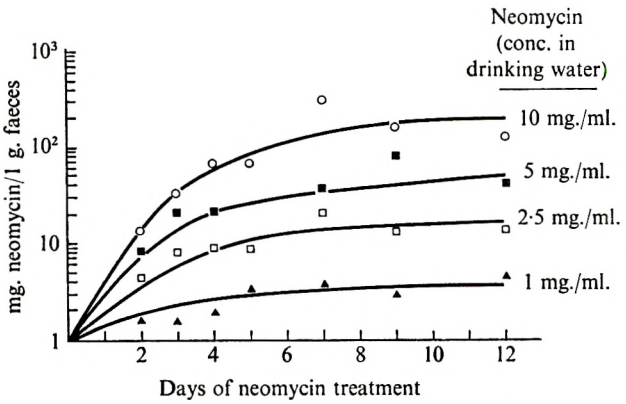

Fig. 1

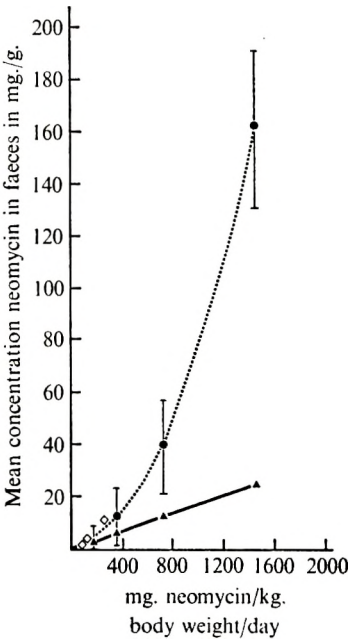

Fig. 2

Fig. 1. Mean concentration of neomycin in the faeces determined for 12 days after the onset of treatment in groups of eight mice treated with different oral doses.

Fig. 2. Mean concentration and S.D. of neomycin in the faeces of mice treated for 12 days as well as the average gentamycin concentration in the faeces of the patient in relation to the oral doses given. ●- - - -●, Mice; ◇- - - -◇, patient (gentamycin concentration in mg./g. of faeces); ▲——▲, calculated concentration of neomycin: $\frac{\text{daily dose per mouse}}{\text{mean weight intestinal contents}}$.

killed on day 5 had persistent positive cultures for *Klebsiella aerogenes*. The group receiving this dose for 7 days had sterile cultures from day 4 of treatment on, while in the animals killed at day 12, one animal remained positive for klebsiella for 1 more day.

The serum concentration of the mice treated with the high oral doses of 1440 mg./kg./day varied between 5 and 9 μg./ml. when they were killed on days 7 and 12. The serum concentration of neomycin in all animals treated with the lower doses was lower than 3 μg./ml. No neomycin could be detected in serum of these mice with the *E. coli* strain used for the serum concentration assay.

The food intake was noticeably reduced in the mice treated with the highest dose of neomycin, while it appeared normal in the others. However, this was not specifically investigated.

Patient

Regardless of the quite high dosing of gentamycin in the first 4 days of treatment, low serum concentrations were found during that period (Fig. 3). Only on day 7 was a peak concentration of 8 μg./ml. seen. Thereafter, the serum gentamycin concentration declined during the period that the patient was treated with 4 g. a

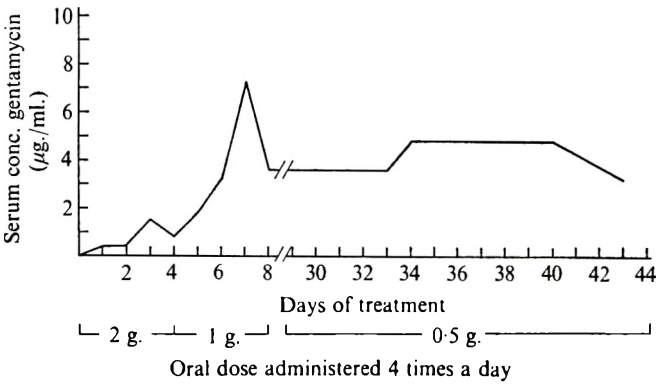

Fig. 3. Serum concentration of gentamycin in the patient during treatment with three different doses.

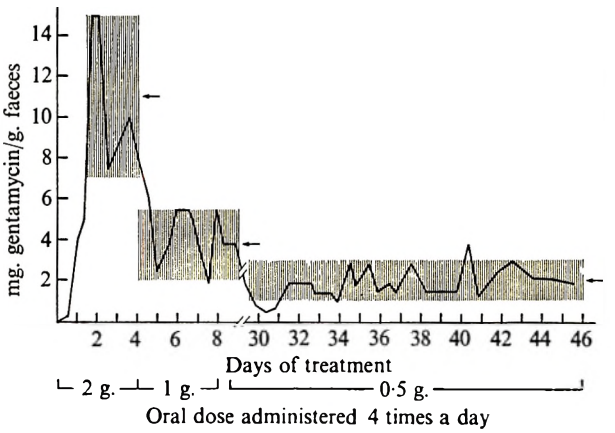

Fig. 4. Gentamycin concentration in the faeces of the patient during treatment with three different doses (the arrows indicate the mean concentrations plotted in Fig. 2).

day to a rather constant value of 4–5 μg./ml. Kidney and liver function tests, performed twice weekly, did not reveal abnormalities during decontamination. The relation between the daily dose of gentamycin per kg. body weight and the average faecal concentration in the patient seems similar to that found in the mice (Fig. 2).

The effect of decontamination of the digestive tract in combination with twice daily bathing in a 0·5 % solution of chlorhexidine (I.C.I.) in water was dramatic. The skin lesions existing at the beginning of the treatment healed in the course of 2 weeks, while there was no formation of new blisters on the skin. The stool cultures were sterile after 48 hr. of treatment and remained so during treatment.

DISCUSSION

The present study indicates that the oral administration of non-absorbable antibiotics for decontamination of the digestive tract of the mouse can safely be started with doses of neomycin as high as 720 mg. per kg. body weight per day (5 mg./ml. of drinking water) if indicated by the sensitivity test. However, the use

of much higher doses seems to be contra-indicated and it is obvious that if treatment is started with the higher doses, the dose should be reduced as soon as possible. As a rule, we decrease the neomycin concentration in the drinking water usually to 2·5 mg./ml. or lower after one week of treatment without recurrence of bacterial growth.

The quite long interval between the beginning of treatment and the establishment of a steady concentration in the faeces in the mouse (Fig. 1) is probably the result of the reduced water intake during the first 3 days of treatment. The enlargement of the caecum, however, which was three-fold in the first week, could also contribute to this effect. In other words, a relatively small dose is suspended in a gradually increasing volume.

The gentamycin concentration in the faeces of the patient reached a plateau on the second day of treatment. A similar relatively rapid build up of another orally administered aminoglycoside antibiotic (kanamycin) in the faeces of man was reported by Cohn (1958). In 12 surgical patients selected on the basis of being in a good general condition and having no lesions in the large bowel, kanamycin reached a steady maximum concentration after one day of treatment. This rapid establishment of the final antibiotic concentration in the faeces may be due to the fact that Cohn's patients were given enemas before treatment, while our patient was not. They were treated with 1 gram every 6 hr. The average faecal concentration was 5 mg./gram. Assuming that Cohn treated adults, this is in agreement with our observations in the patient and the mice.

Woodward, Herrmann & Shadomy (1964) treated conventional rats with a dose of neomycin varying between 160 and 200 mg. per kg. body weight in the diet. It took only 3 days in the rats before the faecal neomycin concentration had reached its final concentration range of 4–4·5 mg. per gram. The difference in build up of the concentration of the antibiotic in the faeces between mice and rats can probably be ascribed to the fact that the rats apparently took the dose of antibiotics from the first day, while the mice had a reduced intake in the first three days. The bitter taste of bacitracin may have contributed to the initial reduced intake.

Another important observation in our study was that the antibiotic concentration in the large bowel following the higher dosages was much higher than was to be expected on the basis of thorough mixing of the daily dose with the intestinal contents (Fig. 2, calculated curve). A daily dose of 720 mg. of neomycin/kg. body weight per day, for example, would then result in an intestinal concentration of approximately 12·5 mg/g. (which is about 25 % of what we found after 12 days of treatment) when the intestinal (colon) contents with an average weight of 2 gram are replaced daily. The exponential rise of the neomycin concentration in the faeces is difficult to explain. Since it has also been observed in monkeys by Hendriks *et al.* (1974), we assume that the antibiotics are inactivated by one or more substances in the intestinal (faecal) contents. It is, for example, known that the antibacterial activity of oligosaccharide antibiotics is decreased by phosphate, citrate, chloride, aluminium and magnesium as well as by other chemicals like cysteine, glutathione, glucose, ascorbic acid, hydroxylamine, semicarbazide, peptone,

and complexes like nucleic acid, thymonucleoproteins etc. (Heilmeyer, 1965). The cephalosporin antibiotics on the other hand are inactivated by protein substances. The consequence of our findings for antibiotic decontamination is that a considerable part of low oral doses will be inactivated. This means that doubling the dose will result in more than double the concentration in the intestinal contents. Also the chemical composition of the diet may be of influence; the more inhibiting substances the greater the loss of antibacterial activity.

The pharmacodynamics of orally supplied nonabsorbable antibiotics are presently under investigation by W. D. H. Hendriks *et al.* in our laboratory. This study is performed in monkeys in order to obtain more detailed information. The monkey was selected since it may be a better model for man than the mouse.

An investigation of this kind can only be performed systemically in experimental animals owing to the fact that administration of high doses of toxic (after absorption) antibiotics which was done in the present study, is not permitted in man without a strict indication. In addition to the pharmacodynamics of oral dosing of 'nonabsorbable' antibiotics, several parameters such as possible toxic effects on remote organs including the liver, kidneys, and bone marrow will be determined.

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Elimination of bacteria from dogs with antibiotics*

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SUMMARY

The effect of oral administration of neomycin cephalothin or kanamycin cephalothin on the aerobic intestinal bacterial flora, was studied in dogs maintained under isolation conditions in a conventional animal room. The dogs were successfully freed of aerobic bacteria with both combinations within two to seven days after the start of antibiotic treatment, and were maintained bacteria free for up to 21 days. Decontamination was attained more rapidly in dogs that were bathed in hexachlorophene surgical soap before and during the first and third days of antibiotic treatment. There was no evidence of toxicity from either of the antibiotic combinations. These results indicate that, as with mice and monkeys, decontamination of dogs with oral antibiotics is feasible. The technique is of potential value in preventing endogenous bacterial infections in canine experimental studies involving use of immunosuppressive agents.

INTRODUCTION

Bacterial infections are a principal cause of morbidity and mortality in conventional experimental animals that have been subjected to immunosuppression (Abaza, Nolan, Watt & Woodruff, 1966). Similarly, after receiving immunosuppressive agents in conjunction with organ transplants or cancer therapy, patients are also highly susceptible to endogenous infections from potentially pathogenic micro-organisms in their microflora (Rifkind, Marchioro, Waddell & Starzl, 1964; Remington, 1972). Protection of patients and experimental animals against such infections is best provided by selectively eliminating the causative bacteria with antibiotics, combined with simultaneous strict isolation to prevent recontamination (van der Waaij & Andreas, 1969; Vossen & van der Waaij, 1972). Protective isolation systems for this purpose are available and are being used increasingly in experimental medicine and medical practice (Levitan & Perry,

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1968; McGarrity *et al.* 1969; Barnes & Tuffrey, 1968; Levitan, Schulte, Strong & Perry, 1967; Dietrich, Fliedner & Krieger, 1973). However, the techniques for selective elimination of potentially pathogenic bacteria with antibiotics are complex; they have been well studied in only a relatively few animal species. Furthermore, the toxicity of certain of the antibiotics could be a limiting factor in their use for the extended periods required to achieve decontamination.

Oral, nonabsorbable antibiotics have been used successfully in mice (van der Waaij & Sturm, 1968; Heit, Wilson, Fliedner & Kohne, 1973) and in monkeys (van der Waaij, de Vries & Lekkerkerk, 1970) to eliminate bacteria from the digestive tract. In mice, coincident with the decontamination of the digestive tract, the skin flora disappeared in the first 4 weeks of antibiotic therapy except for a few bacillus species (van der Waaij & Sturm, 1971). In monkeys, the skin microflora largely disappeared in 48 hours after antibiotic therapy was started (van der Waaij *et al.* 1970). This study was undertaken to determine whether dogs can be similarly decontaminated, and whether specific antibiotics can be administered without toxic effects for the time required to eliminate the aerobic intestinal microflora.

MATERIALS AND METHODS

Animals, animal care, and housing

Ten healthy, mongrel, male and female dogs, estimated to be 6–36 months of age, and weighing 4–14·5 kg. were used. Each dog was isolated and caged individually in a separate conventional animal room. A freshly washed stainless-steel cage, equipped with a sterilized feeder and waterer was provided daily. Sterilized food and water also were provided daily. The principal investigator was the only human contact; a sterile gown and gloves and a face mask were worn whenever the dogs were handled. Three of the ten dogs were bathed with hexachlorophene surgical soap before treatment with antibiotics, and during the first and third days of treatment. They were scrubbed vigorously with a soft-bristle surgical scrub brush for 10 min. and rinsed with tap water.

Antibiotics

Combinations of neomycin-cephalothin, and kanamycin-cephalothin were used. The minimal effective dose of each antibiotic to achieve decontamination of the gastrointestinal tract was determined by a two-phase sensitivity test identical with that described previously for use in monkeys (van der Waaij *et al.* 1970).

The calculated dose of antibiotics was administered orally three times daily in a suspension, in small amounts, over a 10 min. period. In addition, 500,000 units of nystatin (USP) were administered three times daily to control fungi. Nystatin treatment was started 3–4 days before antibiotic treatment was initiated. Previous experience indicated that elimination of yeasts and fungi from the gastrointestinal tract could be accomplished more readily in the presence of the enteric flora. On the first day of treatment, oropharyngeal cultures were taken at 30 min., 60 min., and hourly thereafter for 6 hr. after the antibiotics were administered to determine whether the combination was effective.

Test samples

During the decontamination period, cultures were obtained daily from the skin of the back, feet, perirectum, and chest; from the oral cavity, and from the rectum. Cotton-tipped swabs sterilized in tubes containing 10 ml. brain heart infusion broth were used. After incubation for 24 hr. at 37° C, the cultures were subinoculated on Staphylococcus 110, buffered azide glucose glycerol agar (BAGG), and Endo selective media. The subcultures were incubated for 24 hr. at 37° C. and the results recorded.

Blood and fresh faeces were collected weekly. Blood and serum were examined for changes indicative of antibiotic toxicity. Fifteen ml. of heparinized blood was collected weekly for determination of white blood cells (WBC), red blood cells (RBC), haemoglobin, and blood urea nitrogen (BUN). Total protein, serum glutamic oxalacetic transaminase (SGOT), and alkaline phosphatase were also measured (Table 1). Samples of serum and faeces were frozen at $-20°$ C. for subsequent measurement of antibiotic concentration. Twenty-four-hour bacterial cultures were prepared in final dilutions of 10^5 *Escherichia coli* organisms/ml. in tryptose broth containing tetrazolium (Goss & Cimijotti, 1968). Two strains of *E. coli* were used; a cephalothin resistant-neomycin sensitive strain, and a cephalothin sensitive-neomycin resistant strain. This made it possible to determine the concentration of the antibiotic components separately. Stock dilutions of neomycin and cephalothin were made in concentrations of 100, 50 and 25 mg./ml., and each of these concentrations was serially diluted four steps in 9 ml. of brain heart infusion broth. The bacterial cultures were tested for susceptibility to the antibiotics as follows: 0·05 ml. tryptose broth was added to each well of the microtiter plate; 0·05 ml. of each antibiotic concentration (12 concentrations) was added to two wells in column 1 of the plates; these wells were mixed by to-and-fro rotation of the microdiluters; the microdiluters then were transferred to the well in column 2 and mixed. With the same technique, serial dilutions were made from column 2 through 11; 0·05 ml. of the 10^5 bacterial culture was added to each well in columns 1–11. Column 12 was a media control. The plates were sealed with cellophane tape, incubated aerobically for 24 hr., and examined. The first clear cell in each row was considered the minimum inhibitory concentration (MIC). The results were plotted on a semilog scale and used as the standard for reading the MIC for the test.

The determination of the antibiotic concentration in the faeces and serum was carried out after the samples were thawed. The faecal samples were diluted 1/10 in tryptose broth; the serum was not prediluted. The tests were performed as described above, substituting 0·05 ml. of sample for the antibiotic.

RESULTS

Eight of the ten dogs used in this study were successfully freed of bacteria that could be demonstrated by standard aerobic culture techniques (Table 2). Complete decontamination was achieved 2–7 days after the start of antibiotic treatment. Thereafter, with continuous administration of antibiotics, the dogs were maintained free of bacteria for up to 21 days, except for an occasional contaminant

Table 1. *Haematological and blood chemistry values during antibiotic decontamination*

Dog no.	Days after start of treatment	WBC (10^3/mm.^{3})	RBC (10^6/ mm.^{3})	HB (g./100 ml.)	BUN (mg./ 100 ml.)	Total protein (g./ 100 ml.)	SGOT (I.U.)	Alk. phos-phatase (I.U.)
1	0	18·5	7·6	16·0	15	6·2		
	7	15·2	8·8	16·5	15	6·1	21	28
	14	13·5	7·9	17·0	10	6·2	32	36
2	0	12·8	8·0	17·0	16	7·0		
	7	13·3	8·5	18·0	10	6·2		
	14	13·8	7·9	17·0	15	5·8	28	33
3	0	32·0*	8·1	18·0	15	5·0	40	41
	7	18·7	8·0	17·0	18	6·1		
	14	18·4	8·5	15·6	20	6·2	33	34
4†	0	11·3	7·9	17·0	10	6·2	29	14
	7	18·4	6·8	17·4	15	5·8	26	13
	14	7·6	6·8	15·0	10	6·4	30	34
5	0	11·1	6·1	15·5	15	5·1		
	7	9·8	7·5	11·9	19	4·7		
	14	12·1	5·6	14·0	16	7·3		
6†	0	20·0	8·4	14·5	10	7·2		
	7	21·5	8·4	17·2		7·0	61	75
	14	20·8	7·4	16·4	17·5	6·2	50	147
7	0	12·5	7·7	17·9	3·6	5·6	35	25
	7	13·3	8·0	14·4	9	5·8	78	59
	14	NA‡	NA	NA	NA	NA	NA	NA
8	0	21·6	8·0	16·5	19·8	6·1	50	162
	7	9·9	6·7	15·7	11·2	6·3	52	29
	14	NA	NA	NA	NA	NA	NA	NA
9†	0	6·4	6·8	15·0	17·2	5·4	38	30
	7	6·6	7·1	16·5	16·4	5·3	35	25
	14	5·9	6·7	15·2	28·8	5·1	45	37
10	0	9·9	9·2	17·4	13·0	6·5	25	62
	7	16·0	8·2	17·5	13·4	6·4	21	54
	14	9·8	9·8	17·9	15·8	6·7	31	71

* No clinical evidence of disease or infection was noted in this dog.
† Bathed with hexachlorophene surgical soap.
‡ Not available.

from the room or one introduced by faulty technique. Two dogs remained positive for bacteria throughout the treatment period; one developed resistance to the antibiotic combination, and another inadvertently received less than the calculated dose of antibiotics (Table 2). Age and sex had no apparent effect on decontamination. Weight was a major factor in determining the dose of antibiotic but did not otherwise affect the decontamination procedure.

Decontamination of the three dogs that were bathed with hexachlorophene surgical soap was attained more rapidly than was decontamination of unbathed dogs; two to four days were required but unbathed dogs required from four to seven days to become decontaminated (Table 2). Serum antibiotic concentrations

Table 2. *Decontamination of dogs with antibiotics*

Dog no.	Est. age (months)	Weight (kg.)	Antibiotic combination	Dose of each antibiotic (g. TID)	Total antibiotic concentration		Decontamination status		Days required
					Faeces (μg./g.)	Serum (μg./ml.)	GI tract	Skin	
1	12	9·0	Neomycin-cephalothin	1·0	150	5	C	C	5
2	6	6·0	Kanamycin-cephalothin	0·5	300	8	C	C	4
3	36	14·5	Neomycin-cephalothin	1·0	80	5	I	I	I
4*	12	4·0	Neomycin-cephalothin	0·5	150	5	C	C	4
5	9	6·5	Neomycin-cephalothin	1·5	90	5	I	I	I
6*	36	11·0	Neomycin-cephalothin	2·0	150	8	C	C	2
7	12	9·0	Neomycin-cephalothin	1·5	650	8	C	C	7
8	18	11·0	Kanamycin-cephalothin	1·0	300	5	C	C	6
9*	15	4·5	Neomycin-cephalothin	0·5	150	8	C	C	3
10	12	11·0	Neomycin-cephalothin	1·0	300	5	C	C	7

C = Complete decontamination.
I = Incomplete decontamination.
* Bathed with hexachlorophene surgical soap.

ranged from 5 to 8 μg./ml. Faecal antibiotic concentrations ranged between 150 and 650 μg./g. in the eight dogs in which decontamination was achieved.

A direct relation was found between decontamination of the gastro-intestinal tract and elimination of bacteria from the skin. The bacteria isolated from the skin after treatment had begun were identical with those isolated from the gastro-intestinal tract. Evidence of contamination of the gastro-intestinal tract with normal skin flora such as staphylococci was not found. No changes in blood or clinical chemistry values, suggestive of antibiotic toxicity, were found (Table 1).

DISCUSSION

These results indicate that decontamination of dogs with orally administered antibiotics is feasible. Housing the animals in conventional rooms posed no particular problems when strict hygienic practices were followed. Strict isolation of the animal was deemed essential from the time of sampling the faeces through the decontamination period, to prevent bacterial colonization of the gastrointestinal tract with a contaminant having a different sensitivity pattern. Hexachlorophene surgical soap baths reduced the time for decontamination while the methods of strict isolation and careful handling reduced the chances of recontaminating the skin and hair coat with faecal bacteria.

Neomycin and cephalothin, although classified as nonabsorbable, are minimally absorbed in the dog, as they are in man and monkeys (van der Waaij *et al.* 1970). Neomycin was used to control Gram-positive and certain Gram-negative organisms; cephalothin was used for its broader spectrum of bactercidal activity. Gentamycin, bacitracin and several other antibiotics also are minimally absorbed, but they are more expensive. Oral antibiotics that are nonabsorbed or are only minimally absorbed are preferred, to limit the possibility of immunosuppression stemming from the antibiotic therapy itself.

The faecal antibiotic concentration should be measured at least twice weekly, to provide an index of the minimal concentration needed to inhibit bacterial growth. A continuing concentration of antibiotics in the faeces of at least 100 μg./g. is essential (van der Waaij *et al.* 1970). The dose of antibiotics can be reduced as the faecal bacterial population is reduced, provided the faecal antibiotic concentration does not fall below 100 μg./g.

The study of antibiotic decontamination not only involves bacteriological feasibility, but also its effect on organ functions and possible toxicity. If antibiotic decontamination is applied in organ transplantation, toxic effects of the antibiotics used must be avoided because transplanted organs such as the liver or the kidneys are particular target organs of these antibiotics. It is known from studies in man (Vossen, Dooren & van der Waaij, 1973) and in monkeys (Hendriks, personal communication) that, although so-called nonabsorbable antibiotics are employed for decontamination, toxic alterations in tissue and organ enzymes or in serum proteins may follow oral administration. These alterations can sometimes be measured in serum, and, when properly evaluated, may be an important aid to diagnosis of toxic effects. The antibiotic concentration in serum should be measured

at least weekly because such measurements may give the first indications of approaching toxicity, which should be avoided. Serum concentrations observed during our investigation were between 0 and 8 μg./ml. The exact toxic concentration for dogs needs further investigation; however, values above 15 μg./ml. for neomycin, 25 μg./ml. for kanamycin (Cohn, 1958) and 50 μg./ml. or higher for cephalothin (Venuto, Stein & Ferris, 1972) are usually considered as approaching the toxic range and should be avoided. Toxicity may result from reduced organ function, as in the case of an antibiotic that is normally detoxified by that organ. It may also indicate a dose above the threshold for that organ, or increased absorption from the gastrointestinal tract (Loomis, 1968).

The erythrocyte count and blood chemistry values remained relatively stable in the dogs during antibiotic decontamination, with no consistent changes in any of the values (Table 1). Our observations on the total WBC count show a similar pattern during decontamination to that described for monkeys; total WBC counts in monkeys were shown to decrease slightly after an initial increase, during the first few days of antibiotic treatment (van der Waaij *et al.* 1970). These changes were accompanied by a transitory rise in lymphocytes and a fall in granulocytes. If dog 3 (Table 1) is excluded because of his abnormally high initial WBC count, which we cannot explain, and dogs 7 and 8 are excluded because of the unavailability of WBC counts for day 14, the average counts of the seven other animals at days 0, 7 and 14 also show a peak at day 7. Data about the ratio between lymphocytes and granulocytes during antibiotic decontamination are not available. It seems likely, however, that the higher elevation of the WBC in the first week in the dogs can also be attributed to a rise in the lymphocyte count.

Behavioural and environmental factors can significantly influence the decontamination process. Dogs that walk or rest in their faeces can readily recontaminate themselves as long as bacteria are present in the faeces. Dogs that salivate profusely may dilute and expel the antibiotic before it reaches the base of the oral cavity; others may not permit the antibiotic to remain in the oral cavity for an adequate time. In dealing with this problem we found it helpful to make a suspension of the drugs and to administer the suspensions in small quantities over longer intervals until the calculated dose was given. Dogs requiring longer periods for decontamination were believed to have had less than an adequate dose of antibiotics or less than adequate contact time with the antibiotic.

The feasibility of this technique suggests that it could usefully be applied in preventing endogenous bacterial infections such as may occur in experimental studies involving immunosuppression (Abaza *et al.* 1966).

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Staphylococci in swimming pool water

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SUMMARY

During a period of five years 1192 water samples from swimming pools were examined for staphylococci and 338 for coliform organisms only. Eighty-nine different pools were sampled.

Numbers of staphylococci, estimated by the membrane filtration technique did not bear any significant relation to either bathing load or concentration of free chlorine.

Wide variation in the staphylococcal count was observed when different parts of a pool were sampled on the same occasion.

The only practicable standard for pool samples in relation to staphylococci would appear to be that these organisms should be absent from 100 ml. water when the pool has been out of use during at least ten hours before sampling if filtration and chlorination are adequate.

INTRODUCTION

There have been several suggestions that coliform organisms were not the best indicators of bacterial quality of chlorinated swimming pool waters (Victorin, 1974). Determination of staphylococci in particular was advocated by Ferramola & Elena Durieux (1951) but they neither made counts nor reported free chlorine concentration. Robinton, Mood & Elliot (1957) suggested examining a sample of 100 ml. using a membrane filter for cocci; they found that staphylococci predominated in samples with low residual chlorine and suggested that estimation of free chlorine was a sufficient test for safety because of the rapid rate of kill with a high concentration. A Report (1953) by the Public Health Laboratory Service Water Subcommittee concluded that staphylococci were too resistant to chlorine to be satisfactory indicator organisms. Favero, Drake & Randall (1964) advocated the use of staphylococci as indicators of pool pollution and proposed a standard of fewer than 100 staphylococci per 100 ml. water. Keirn & Putnam (1968) considered 30 staphylococci/100 ml. in less than 15 per cent of samples to be a realistic standard. Robinton & Mood (1966) showed that bathers shed staphylococci most consistently and in the largest number compared with other organisms. Villa & Zaffino (1970) concluded that no single test was adequate but that both coliform

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organisms and staphylococci should be looked for concurrently in assessing the results of chlorination of pool water samples. Barnard (1972) examining samples from a hydrotherapy pool found a high count of Gram positive cocci whenever bathers were in the water whatever the free chlorine concentration.

This paper describes an investigation to determine whether enumeration of staphylococci in chlorinated swimming pool water provides a reliable indication of swimming pool contamination which might be used as a guide to management of water treatment.

METHODS

Chlorine estimations were made at the pool side by using the diethyl-*p*-phenylene diamine (DPD) test (Palin, 1957). Samples for culture were taken about two inches below the surface in sterilized bottles containing sodium thiosulphate and transported immediately to the laboratory. Presumptive coliform organisms were estimated by the multiple tube method using MacConkey broth with Teepol 610 substituted for bile salts. Staphylococcal counts were made by the membrane filtration technique described in Report (1969) using Oxoid staphylococcal medium 110 with addition of bromo-thymol blue, membranes being incubated for 48 hr. at 30 °C.

RESULTS

During 1967, between May and September, 227 water samples from 20 swimming pools of various types were examined both for most probable number (MPN) of presumptive coliform organisms and for numbers of staphylococci. Seventy-eight samples (34 %) were found to contain neither coliform organisms nor staphylococci; 60 samples (26 %) contained both and 84 samples (37 %) contained staphylococci but no coliform organisms. Five samples (3 %) yielded coliform organisms but no staphylococci. Thus 144/227 samples (63 %) contained staphylococci. Numbers of staphylococci were less than 100/100 ml. in 62 samples (43 %) and 100 or more/100 ml. in 82 (57 %) samples. Of staphylococci isolated 65 % were found to be coagulase positive.

In 1968 a study was made of the distribution of coliform organisms and staphylococci in 133 samples grouped according to total (free and combined) chlorine concentration. In 44 samples (33 %) neither coliform organisms nor staphylococci were found, a figure close to that of 1967. Table 1 shows the results. It will be noted, summing appropriate entries of the table, that staphylococci were detected in 84/133 samples (63 %), a figure identical with that obtained in 1967, but only 21/37 (57 %) samples with 1·0 parts per million (ppm) or more total chlorine contained staphylococci whereas 41/56 (73 %) samples with less than 0·5 ppm did so. In only five samples (3·8 %) were coliform organisms found in the absence of staphylococci, all these samples being taken from pools which were out of use and whose water received no treatment.

In 1969 an investigation of coliform organisms, including *Esch. coli*, in relation to the presence of bathers at the time of sampling was made. Table 2 shows results on 338 samples grouped according to the free chlorine concentration from which it

Table 1. *Numbers of samples showing association between coliforms and staphylococci in three ranges of chlorine concentration. Results of* 1968

	Total chlorine (ppm)			
	< 0·5	0·5–0·9	1·0 and above	
Coliforms absent Staphylococcus absent	13	17	14	44
Coli absent Staphs present	21	15	16	52
Coli present Staphs present	20	7	5	32
Coli present Staphs absent	2	1	2	5
Totals	56	40	37	133

Table 2. *Numbers of samples with coliforms and* E. coli *according to the presence of bathers and two ranges of chlorine concentration. Results of* 1969

	Free chlorine < 1·0 ppm		Free chlorine ⩾ 1·0 ppm		
	No bathers	Bathers present	No bathers	Bathers present	Totals
Total samples	78	114	54	92	338
Samples with coliforms	29	45	8	8	90
Samples with *Esch. coli*	6	22	0	0	28

can be seen that among 192 samples with free chlorine of less than 1·0 ppm 74 (38·5 %) yielded coliform organisms and 28 (14·5 %) *Esch. coli*, whereas 146 samples with free chlorine of 1·0 ppm and over yielded coliform organisms in only 16 (10·9 %) but none proved to be *Esch. coli*. It is evident that, as expected, coliform organisms are reduced in number by increasing free chlorine concentration. From Table 2 it can also be seen that of 206 samples taken when bathers were present 53 (25·7 %) yielded coliform organisms and 22 (10·7 %) *Esch. coli*. Samples taken when no bathers were present numbered 132 of which 37 (28 %) yielded coliform organisms and only 6 (4·5 %) *Esch. coli*. It can be concluded that the presence of bathers at the time of sampling increases the frequency of isolation of *Esch. coli* if the free chlorine concentration is less than 1·0 ppm.

In 1970 samples from 89 pools, including those previously sampled, were examined by the staphylococcal count only and classified according to the free chlorine concentration and the number of bathers using the pool at the time of sampling to determine whether numbers of staphylococci in samples were associated with a low chlorine concentration or with the presence of bathers (Tables 3 and 4). These pools were of all varieties of size and chlorination methods but all were filtered. The best straight line of multiple regression fitted to the data of Table 3 was:

count of staphylococci = 22·1 − 1·67 × chlorine concentration + 4·56 × presence

Table 3. *Some results of* 1970 *showing numbers of samples with fewer than* 100 *staphylococci per* 100 *ml. according to presence of bathers and amount of free chlorine*

Staphylococcal count (mean of range)	Chlorine		Bathers	
	Low (< 1 ppm)	High (≥ 1 ppm)	Absent −	Present +
0	28	32	35	25
5	45	55	58	42
15	19	25	26	18
25	8	15	10	13
35	14	5	11	8
45	8	9	8	9
55	6	2	4	4
65	5	7	5	7
75	5	7	6	6
85	5	7	6	6
95	4	3	3	4
Total	147	167	172	142

Table 4. *Some results of* 1970 *showing mean counts of* 87 *samples with* 100 *and more staphylococci per* 100 *ml. and chlorine* ≥ 1 *ppm.*

Staphylococcal mean count	Chlorine			
	1	1·5	2·5	3·5
	Number of samples			
302	—	—	—	2
378	—	—	9	—
456	50	—	—	—
530	—	26	—	—

of bathers. Neither 1·67 nor 4·56 differs significantly from zero (t = (respectively) 0·06 and 0·17 and would have been exceeded by chance in 80–90 % of trials).

Table 4 shows the number of samples of which the chlorine concentration was high and the mean staphylococcal count 100 or more per 100 ml. Thirty-seven samples with 1·5 or more ppm chlorine concentration gave a mean count of 302 or more staphylococci. The best straight line was:

count of staphylococci = $466{\cdot}5 - 42{\cdot}6 \times$ chlorine concentration. 42·6 does not differ significantly from zero (t = 0·11 and would be exceeded by chance in 90 per cent of trials).

From these two sets of findings it can be concluded that the association of staphylococcal numbers with absence of bathers and concentration of chlorine was not significant.

In 1971 samples examined numbered 431 (Table 5) of which 156 contained less than 1·0 ppm free chlorine and 275 over this amount. In the group with less than 1·0 ppm there were 67 samples. (42·9 %) containing fewer than 10 staphylococci per 100 ml. whereas in the group with 1·0 ppm and over 148 (53·8 %) contained fewer than 10 staphylococci/100 ml. Of 75 samples yielding 100 staphylococci or

Table 5. *Showing distributions of counts of staphylococci at low and high free chlorine concentrations of samples of* 1971. *Number of samples at each range.*

Count (mean of range)	Free chlorine	
	(< 1ppm)	(≥ 1 ppm)
0	33	75
5	34	73
15	14	27
25	10	21
35	3	13
45	0	10
55	5	4
65	3	5
75	5	5
85	3	1
95	4	8
137	16	14
277	10	6
43	6	6
705	6	1
1292	4	6

over/100 ml. 42 had less than 1·0 ppm and 33 had more than this concentration of free chlorine. The regression line fitted to all the results of Table 5 was:

count of staphylococci $= 148 - 52{\cdot}4 \times$ concentration of chlorine. 52, unlike the regression of Tables 3 and 4, differs significantly from zero ($t = 2{\cdot}35$ and $0{\cdot}02 > p > 0{\cdot}01$).

Variability amongst samples

The results so far obtained were calculated as the mean of counts on two samples of pool water taken from different places, usually the deep and shallow ends, on the same occasion.

To assess the variability of staphylococcal numbers in different parts of a pool at the same time, two pools (A and B) were examined by taking three samples on each occasion, bathers being absent. Results were highly variable indicating a far from homogeneous staphylococcal distribution probably due to aggregation of organisms on skin squames and in mucus. It will be seen (Table 6) that on 12 occasions the count at the inlet was higher than counts at the middle and outlet. The total variances of the numbers of staphylococci in pools A and B were analysed into those ascribable to position in the pool, (α) those to occasion of sampling (β) and those to error (γ). For neither pool were the variance ratios α or β divided by γ significant at the 5% point; indeed for pool A (Table 6) variance due to γ exceeded that due to α and β. When samples were taken from pool A before use by the first bathers of the day, after water circulation through the filters and chlorination had continued all night, it was found that staphylococci were present, if at all, only in small numbers (5/100 ml.). When the free chlorine was raised to 1·0 ppm no staphylococci were found after all night water treatment during 10 hours or more. Similar results were obtained with other pools.

Table 6. *Counts of staphylococci on 32 sampling occasions and three places at Pool A*

Inlet	Middle	Outlet	Inlet	Middle	Outlet
0	564	29	14	1	0
103	5	61	24	64	0
0	4	1	13	66	86
25	79	55	4	4	5
0	0	0	38	5	6
13	42	0	0	6	0
8	28	9	12	10	4
0	0	3	0	5	64
1	2	5	350	1	1
10	0	0	13	66	86
60	12	18	36	45	57
0	48	400	7	1	9
152	8	7	7	19	114
48	191	46	140	64	87
106	18	21	332	25	54
0	0	0	6	4	0

DISCUSSION

A review of the literature reveals that the use of swimming pools is occasionally associated with subsequent illness. Hellerström (1951) reported skin nodules containing acid-fast bacilli occurring after small abrasions while bathing in swimming pools. There have been later accounts of granulomata following injury by the rough sides of pools (Tolmach & Frank, 1953; Thomas, 1967). In the Netherlands, Sluyter (1973) found tuberculin conversion in children associated with use of a swimming pool from which Runyon group IV organisms, but not *Mycobacterium balnei*, were isolated. English & Gibson (1959) found swimming pools an important source of tinea pedis infection. Plantar warts occurred more commonly among children using heated, covered pools than among users of other types of pool (Allen & Dickinson 1968). Gentles & Evans (1973) showed that both verruca and tinea pedis infection spread among the users of a covered swimming pool. In the U.S.A. Bell and co-workers (1955) investigating outbreaks of conjunctivitis in summer camps found the incidence 50% greater among users of camp pools than among non-users. It has been long established that inclusion conjunctivitis is associated with the use of swimming pools, the TRIC agent responsible, a member of the *Chlamydia* (*Bedsonia*) genus, evidently being initially derived from vaginal or urethral discharges of bathers and subsequently from conjunctival washings of those infected. Non-infective conjunctivitis may be caused by combined chlorine. Ormsby & Aitchison (1955) in Canada reported pharyngoconjunctival fever spread by swimming pool use. van der Veen & van der Ploeg (1958) associated adenovirus infection with swimming in an unchlorinated pool as did Kallings & Madsen (1961) and Foy, Cooney & Hatlen (1968). McLean (1963) isolated parainfluenza 1 virus from swimming pool water. Clarke, Stevenson & Kabler (1956) concluded that transmission of adenovirus in a 'well chlorinated' pool was unlikely because water without demonstrable coliform organisms was

probably free from adenovirus. Anderson & Jamieson (1972) described a case of meningoencephalitis due to *Naegleria fowleri* in swimming pool water from which the organism was not eradicated by chlorination to 10 ppm.

Epidemiological evidence is slowly accumulating that bathers using badly managed pools are at risk from cross-infection. It must be remembered that even if pool water is of satisfactory standard the floors of the pool surround and changing rooms must also be kept at a high standard of cleanliness.

Results reported here show that staphylococci, of which about 65 % are *Staph. aureus*, are always present in water of a pool in use if coliform organisms are also present and that their numbers are unlikely to be a useful indication of the hygienic condition of a pool. The number found was associated in the expected way with the absence of bathers and the presence of chlorine, both of which might be regarded as justifiable measures of the hygiene of a pool, but for two sets of results the regression was not significant (Tables 3 to 5). It is clear that counts from one or two samples, which are likely to be submitted in practice, could never be used for a judgement. This view is reinforced by the results from samples of pools *A* (Table 6) and *B* which show that no significant part of their variance could be ascribed to situation in the pools or occasion of sampling. However, it was observed that staphylococci were absent from 100 ml. samples when a pool had been out of use during the previous 10 hr. if filtration and chlorination were adequate, that is to say the water circulation time was not more than 4 hours with free chlorine maintained at 1·0 ppm by breakpoint chlorination. Used in these circumstances, a count of staphylococci might be useful in detecting poor filtration, inadequate chlorination or prolonged circulation time since, when these are satisfactory, staphylococci are absent after ten hours treatment. Otherwise our results confirm the conclusion of the Public Health Laboratory Service Water Subcommittee (Report, 1953) that staphylococci are too resistant to chlorine to be useful indicator organisms in examination of the water of swimming pools.

No evidence of illness attributable to the use of the pools examined was found during five seasons of the investigation. Phage typing of 48 strains of *Staph. aureus* yielded types in all three groups but none were resistant to both penicillin and tetracylcine.

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Epidemiology of human brucellosis in Isfahan, Iran*

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SUMMARY

Studies on brucellosis were carried out to elucidate the epidemiology of the disease in Isfahan province, where *Brucella melitensis* is highly prevalent in animals and man. A positive milk ring test of 32 % and 23 % was found in unvaccinated goats and sheep respectively. Card and tube agglutination tests showed an infection rate of about 12 % in sheep and goats and 42 % in cattle. *B. melitensis* was isolated from 8 % of 677 samples of fresh cheese examined.

Of 1526 clinically suspected human cases, 476 showed laboratory evidence of brucellosis. Of these patients, 291 cases were from urban and 185 cases from rural areas. Cases from urban and rural areas were seen principally in the younger age groups. The median age of infection was 19·7 in urban and 15·7 in rural patients respectively. The infection was encountered mainly from April to August. This correlates with animal parturition and the greatest amount of sheep and goat milk production, which is introduced to the local market as fresh cheese. Raw dairy product consumption is the most probable way of *Brucella* transmission in urban patients. In rural areas, both dairy product consumption and contact with animals are sources of infection.

INTRODUCTION

Sheep and goats are the principal farm animals in Iran, and more than 50 million of these animals are raised in the country (FAO-WHO-OIE, 1972). A few organized dairy farms and breeding units are found around the large cities where pasteurized dairy products are consumed. Brucellosis among farm animals is widespread in most parts of the country and, in consequence, human brucellosis is common, especially in areas of high sheep and goat concentration.

Isfahan, located in the central part of Iran, is an agricultural province. Because of its climate and relatively rich pastures, approximately one-third of the country's total number of sheep and goats graze in this area. Animal and human brucellosis is widely prevalent in certain areas of Isfahan and of 4738 cases of human brucellosis in Iran reported to the World Health in 1964, 3811 (80 %) were from this region (Ministry of Health, 1965).

An epidemiological study of human brucellosis was carried out among the rural

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and urban population in Isfahan province. This report covers an analysis of case histories in the areas studied together with the prevalence of animal brucellosis and contamination of dairy products with *Brucella* organisms for the period 1967–1972.

MATERIAL AND METHODS

Clinically suspected human cases from outpatient clinics in the city of Isfahan and from seven villages on its outskirts were brought to the attention of the investigation team by attending physicians. Laboratory diagnosis of brucellosis was based on card, tube agglutination tests and blood culture. Complement fixation tests were performed on those with negative or non-significant agglutinin titre by the above tests. A titre of 1/80 (160 i.u.) or greater by tube agglutination and 1/20 by complement-fixation test was considered positive.

Patients were interviewed for clinical symptoms, and epidemiological history forms were completed.

Unvaccinated sheep, goats and cows with a history of abortion and some animals belonging to the patients were tested for brucellosis. Milk ring, card and tube agglutination tests, as well as blood culture, were employed for the diagnosis of animal brucellosis.

Fresh cheese, cream, ice-cream and butter were collected from retail shops and distribution centres and cultured for *Brucella* isolation. Three recommended antibiotics (Alton & Jones, 1967) were added to trypticase soy agar, and this medium was used for the testing of dairy products. Trypticase soy agar plates were inoculated in duplicate with 10 mg. each of the sample and incubated at 37° C. One plate from each sample was incubated in 10% CO_2 atmosphere. The plates were discarded as negative after 10 days. Identification of *Brucella* strains isolated from human patients, animals and dairy products was made by sensitivity, physiological and serological methods.

RESULTS

Human brucellosis

Of 1526 suspected cases in the areas studied, 476 (31·2%) showed laboratory evidence of *Brucella* infection. Of these patients, 291 cases were from the city of Isfahan and 185 from the villages studied. All of those positive on card test were also positive by tube agglutination test at 160 i.u. of *Brucella* agglutinin or greater.

Seasonal distribution

The seasonal distribution of all cases is shown in Fig. 1. It can be seen that cases occurred throughout the year except during January. About 84% of the cases occurred during the period April–August. There was no difference between urban and rural cases in this respect. The attack rate during the 12-month period corresponded to sheep and goat parturition and milk production.

Age and sex

The age and sex distribution of clinical cases is shown in Table 1. This table indicates that brucellosis in urban and rural areas is principally a disease of the

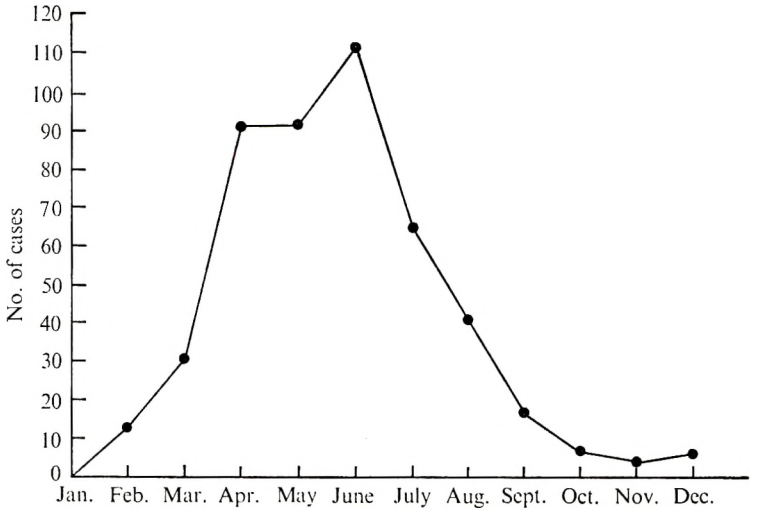

Fig. 1. Seasonal distribution of 476 brucellosis cases in Isfahan, Iran.

Table 1. *Age and sex distribution of 291 urban and 185 rural cases of brucellosis*

	Urban cases				Rural cases			
	Sex				Sex			
Age	M	F	Total	% of Total	M	F	Total	% of Total
0–4	4	1	5	1·7	7	4	11	5·9
5–9	16	10	26	8·9	14	18	32	17·3
10–14	35	25	60	20·6	26	19	45	24·3
15–19	47	11	58	19·9	13	19	32	17·3
20–24	12	9	21	7·2	7	5	12	6·5
25–29	11	11	22	7·6	3	6	9	4·9
30–34	13	4	17	5·8	5	4	9	4·9
35–39	12	5	17	5·8	5	6	11	5·9
40–44	12	11	23	7·9	2	4	6	3·2
45–49	5	8	13	4·5	5	1	6	3·2
50–54	4	10	14	4·8	4	2	6	3·2
55–59	1	1	2	0·7	3	—	3	1·6
60+	7	6	13	4·5	2	1	3	1·6
Total	179	112	291		96	89	185	

younger age groups. Of 291 urban cases, 149 (51·2%), and of 185 rural cases, 120 (64·8%), were found in the age group up to 20. This table also shows that the median age of infection for males and females in the city of Isfahan is 18·6 and 25 years respectively, while the median age of infection for both sexes in rural areas is about 15 years. In general, the median age of infection in urban areas is 19·7 and in rural areas 15·7.

Dairy product consumption

The consumption of five dairy products is shown in Tables 2 and 3. These data have been compared with 250 suspected cases with no *Brucella* agglutinin in

Table 2. *Reported consumption of five dairy products among outpatients from urban areas*

	No. inter-viewed	Fresh cheese (%)	Butter (%)	Raw milk (%)	Ice-cream (%)	Cream (%)
Serologically positive	291	91	59·8	18·5	46·7	26·1
Serologically negative	250	83·2	70·4	16·8	46·8	33·6
Total	541	87·4	64·7	17·7	46·8	29·6

Table 3. *Reported consumption of five dairy products among outpatients from rural areas*

	No. inter-viewed	Fresh cheese (%)	Butter (%)	Raw milk (%)	Ice-cream (%)	Cream (%)
Serologically positive	185	94	66·5	51·9	45·9	50·8
Serologically negative	250	98·8	66	13·2	12·4	30
Total	435	96·8	66·9	29·6	26·7	38·8

Table 4. *Correlation of occupation and reported animal contacts of cases of human brucellosis in urban and rural areas*

	Urban cases		Rural cases	
Occupation	No. of patients	No. reporting animal contact	No. of patients	No. reporting animal contact
Housewives	66	14	40	21
Farm workers	22	9	23	19
Butchers	1	1	2	2
Shepherds	—	—	10	10
Students	62	6	36	18
Tradesmen	15	3	34	7
Labourers	18	4	13	6
Pre-school children	20	—	27	22
Total	204	37	185	105

each of the groups studied. In rural areas, the main difference between the two groups is seen in those who consumed raw milk, ice-cream and cream (Table 3). Table 2 shows that dairy products are consumed equally by the two groups in the city of Isfahan. In general, the percentage of dairy product consumption, especially of fresh cheese, by all interviewed persons in both areas was very high.

Occupations and reported animal contacts

The occupations and reported animal contacts of urban and rural brucellosis cases is shown in Table 4. This table indicates that housewives and students are more affected in both urban and rural groups. About 56% of the patients in the villages reported animal contact while in the city only 18% had animal contact.

Table 5. *Chief symptoms of* 476 *cases of human brucellosis in Isfahan*

Symptom	No. of cases	% of total
Fever	366	76·9
Sweating	375	78·8
Joint pain & arthralgia	454	95·4
Headache	288	60·5
Fatigue	177	37·2
Cachexia	235	49·4
Chills	202	42·4
Constipation	119	25·0
Diarrhoea	7	1·5
Anaemia	268	56·3
Orchitis	36	7·6
Muscle pain	92	19·3
Total	476	

Table 6. *Results of milk ring and tube agglutination tests*

Type of test	Kind of animal	Number tested	Percent positive
M.R.T.	Sheep	1850	23
	Goats	1340	32
Tube agglutination	Sheep	1241	12·7
	Goats	736	12·2
	Cattle	326	42·3
	Camels	1	1/1

Brucella isolations

Positive blood cultures were obtained from 40 out of 123 acute brucellosis cases. Two patients still had a positive blood culture after their treatment was completed and the clinical symptoms had improved. All 40 strains isolated were *Brucella melitensis* biotype I, the species commonly found in sheep and goats and detected continuously in dairy products. *B. abortus* was not isolated from humans on any occasion during this study.

Clinical symptoms

The predominant symptoms in all brucellosis cases were pain in the joints, arthralgia, sweating, fever and headache (Table 5). Anaemia was seen but, because of parasitic infestation, is considered common in this area. More than one half of the cases reported symptoms lasting two months or more and 38 patients were ill for over a year.

Animal brucellosis

Sheep, goats, cattle and camels were tested for *Brucella* infection. Nearly all animals tested were from areas whose products are sold mainly in the city of Isfahan. Table 6 shows the results of milk ring and tube agglutination tests. Card and tube agglutination showed close agreement and a titre of 1/40 (80 i.u.) or greater was considered positive. In Table 7 the results of milk and blood culture are shown. One of the two strains from cow's milk and all of those from sheep and goat milk were *B. melitensis* biotype I. The other strain from cow's milk was *B. abortus* which was not typed.

Table 7. *Results of milk and animal blood culture*

Material examined	Kind of animal	Number of cultures	Number positive
Milk	Sheep and goats	115	3
	Cows	83	2
Blood	Cattle	72	0

Table 8. *Results of four dairy product cultures*

Dairy product	Number cultured	Number positive
Cheese	677	56 (8·3)
Cream	160	1
Ice-cream	295	0
Butter	146	0

Dairy product culture

In the examination of dairy products *B. melitensis* was isolated from a high percentage of cheeses examined (Table 8). Out of 677 fresh cheese specimens collected from retail shops and distribution centres, 56 (8·3 %) were infected with *B. melitensis* biotype I. Only one isolation was made from 160 cream samples and all 295 ice-cream and 146 butter specimens were negative. The number of *Brucella* organisms per gram of cheese was calculated and ranged from 1000 to 131,000 with a mean of 17,750.

DISCUSSION

B. melitensis type I was isolated from human, animal and dairy products. Bovine brucellosis due to *B. abortus* is prevalent in the areas studied, but this organism could not be isolated from human patients. On the other hand, *B. melitensis* type I was isolated from a dairy cow. This indicates that, in an area where sheep and goat brucellosis is endemic, cattle could be one of the animal reservoirs for *B. melitensis*.

The consumption of fresh white cheese, which is produced from unpasteurized sheep and goat milk, is very popular in Iran. This cheese is often manufactured rapidly by adding dried rennin from young goat and sheep abomasum to milk, and is then consumed within a few days. In a previous study (Sabbaghian, 1973) *Brucella* organisms were isolated from cheese four weeks after collection from retail shops.

With the high prevalence of brucellosis in sheep and goats and the contamination of fresh cheese with *B. melitensis*, the high consumption of this dairy product (87·4 %) seems to be the most important source of human infection. Raw milk, although consumed by only 17·7 % of the interviewed group, is another possible source of infection.

Nicoletti & Amini (1971) and Sabbaghian, Ghiasseddin & Abolhassani (1973) have shown that in large cities in Iran, brucellosis is transmitted mainly through the consumption of dairy products. In another study, Feiz, Sohrabi & Sabbaghian (1972) have shown that animal contact is the main source of infection in a village

near Isfahan. The results of this study support the previous findings and indicate that in the city of Isfahan, brucellosis is transmitted mainly through the consumption of dairy products. Although 18% of the urban patients reported animal contact, most of these were from the suburbs of the city and usually maintained a few farm animals. In these cases, infection through animal contact cannot be ruled out.

In contrast to the urban situation, in rural areas about 56% of the patients reported animal contact. On the other hand, a considerable number of the people interviewed in villages reported consumption of unpasteurized dairy products. These findings suggest that, among the rural population in Isfahan province, both contact and non-contact brucellosis occurs.

Bothwell (1960) pointed out that, when the source of infection is dairy products, younger age groups are more frequently affected because of the high rate of dairy product consumption among these groups. In this study, we found that 51·2% of the confirmed urban brucellosis cases were in the age group of up to 20 years. Among the confirmed rural brucellosis cases, 64·8% were in the age group up to 20 years; this may be a result of possible infection by dairy products as well as by animal contact. In the urban and rural population, younger age groups are at a high risk of infection due to unpasteurized dairy products. In addition, the rural young groups are exposed to animal contact sources. In villages, most families keep some kind of farm animals in their own houses and children start working when they are about 7 years of age. Looking after the animals as shepherds is one of the main jobs for children in their spare time. Table 4 shows 50% of reported animal contacts among rural students.

A significant difference between male and female cases of brucellosis in the city of Isfahan could mislead us to suggest that the problem may be occupational. In fact this is not so; only 18% of the urban cases reported animal contacts, and consumption of dairy products is alike for both sexes in this country. A possible reason for the higher rate of brucellosis in males among the urban population may be that more males seek medical care and thus their number appears greater in reports compared with females, as is common in developing countries.

In this study group, housewives without animal contact formed about 25% of the total urban cases, and adult male infection was not higher than female; this suggests that the disease is not occupational in the city of Isfahan.

It has been reported (Boycott, 1964; Report, 1968) that occupational brucellosis is most commonly seen in males. This may not be applicable in all parts of the world, especially where jobs are not well oriented. In developing countries, women work on farms and the chance of coming in contact with farm animals is equal in both sexes. In our study, 21·6% of all rural cases were housewives and about 50% of them reported animal contact. This high percentage report of animal contact among housewives is indicative that, in the rural situation in Isfahan province, occupational transmission of brucellosis is not always confined to men.

In conclusion, in the city of Isfahan most brucellosis cases are transmitted directly through consumption of unpasteurized dairy products, but in the suburban villages both direct and indirect transmission occur.

This study shows that animal brucellosis is prevalent in Isfahan province and, since brucellosis is a zoonosis, control of the disease in animals would result in the reduction of human cases. The production of pasteurized dairy products and public education to elucidate the danger of consuming unpasteurized milk or cheese is recommended for control of human brucellosis.

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The effect of repeated vaccination in an enzootic foot-and-mouth disease area on the incidence of virus carrier cattle

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SUMMARY

A comparison was made of the incidence of foot-and-mouth disease virus 'carrier' cattle in an unvaccinated enzootic area and an area where routine 6-monthly vaccination with an inactivated vaccine had been carried out for 3–4 years. The incidence of carriers in the vaccinated area was 0·49 % as compared to 3·34 % in the non-vaccinated area. The results indicate that, provided the immune status of the vaccinated herd is maintained at a level sufficient to prevent outbreaks of clinical disease and the re-introduction of virus is prevented through livestock movement controls, it should be possible to eradicate the disease from an enzootic area through vaccination.

INTRODUCTION

Traditional methods used for the control of outbreaks of foot-and-mouth disease in enzootic areas have included either aphthization or vaccination of all in-contact susceptible cattle and vaccination of uninfected farms around the outbreak, together with quarantine procedures and control of livestock movement. These methods are successful in containing the disease but do not necessarily help towards eradicating it. Considering that prophylactic vaccination might give better results, in 1968 Kenya embarked on a 6-monthly vaccination programme of all cattle in a limited part of the country. The area was gradually expanded so that by the end of 1972 most of the large-scale farming areas of the highlands had been included. The object was to attempt to eradicate the two main endemic types of foot-and-mouth disease, O and A, by combining routine 6-monthly vaccination, using a bivalent inactivated vaccine†, with the normal rigid livestock movement controls. That disease control was being achieved was apparent from the decrease in the number of clinical outbreaks in the area. During the period 1965–7 there was an average of 52 farms affected per year, while in the years 1968–73 the average had fallen to ten farms per year, with the number steadily decreasing each year. The occurrence of clinical outbreaks does not necessarily give a true assessment of the amount of virus in the environment as subclinical or inapparent infection could

* On secondment from the Animal Virus Research Institute, Pirbright, Surrey, England.
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† Wellcome Foundation.

occur, particularly in partially immune cattle. Van Bekkum, Frenkel, Fredericks & Frenkel (1959) demonstrated the prolonged carriage of virus by animals recovered from the disease by collecting oesophageal and pharyngeal fluid and scrapings. The incidence of such virus carrier animals would give a better indication of whether the vaccination programme was eradicating the virus or merely controlling the incidence of clinical disease. It was for this reason that this survey was undertaken and the preliminary results are reported here. The incidence of 'carriers' in the compulsory vaccinated area is compared with that in non-vaccinated areas of the country.

METHODS

It was shown by Burrows (1966) that the sites in the upper respiratory tract where foot-and-mouth disease virus persists after clinical infection were the pharynx and the dorsal surface of the soft palate. Accordingly, samples were collected from these tissues.

Collection of oesophageal–pharyngeal scrapings

Pharyngeal scrapings from mature steers from the vaccinated areas were collected immediately after slaughter in the abattoir. The retro-pharyngeal wall and lymphoid tissue were scraped with a curette and the scrapings placed in 5 ml. phosphate buffered saline (PBS), pH 7·4, containing 0·5 % (w/v) gelatin, 200 units penicillin, 200 units polymyxin B, 150 g. neomycin sulphate and 50 units mycostatin per ml. The curette was disinfected between samplings by washing in 0·002 % (w/v) citric acid and rinsing in tap water. The samples were kept at 4° C. immediately after collection and transferred to a −20° C. refrigerator within 3 hr.

Oesophageal–pharyngeal (O–P) scrapings from cattle in the non-vaccinated areas were collected by means of a probang, as described by Sutmöller & Gaggero (1965). After collection, the sample was placed in 5 ml. 0·08 M phosphate buffer, pH 7·2, containing the same concentrations of antibiotics as the PBS described above. The sample was kept in dry ice until returned to the laboratory, when it was again transferred to a −20° C. refrigerator. The probang was again disinfected after each animal in 0·002 % (w/v) citric acid and then rinsed in tap water.

Examination of the oesophageal–pharyngeal scrapings for the presence of foot-and-mouth disease virus

All the samples were examined for the presence of virus as soon as possible after collection. A 0·2 ml. volume of each sample was inoculated onto each of five monolayers of primary bovine thyroid cells (Snowdon, 1966) in test tubes. These were examined over a period of 72 hr. and the supernatant from any monolayer showing signs of cytopathic effect (CPE) was collected and passaged on to a further five monolayers. If CPE again developed, the presence of foot-and-mouth disease virus in the culture was confirmed by typing in a micro complement-fixation test. The infectivity of a proportion of the positive O–P samples was determined by titration in primary bovine thyroid cells.

Antibody assay

Sera were collected at the same time as the pharyngeal scrapings and their antibody concentrations to types O and A viruses were assayed in a micro metabolic inhibition test (P. Dawe, personal communication). For this test, modified Eagle's medium containing 4% normal bovine serum, 10% tryptose phosphate broth, 0·3% sodium bicarbonate, 0·2% glucose and 0·004% phenol red was used. This medium was used as the diluent for serum, virus and cells alike. The virus strains used were the same as those used in the vaccine and the cell substrate was baby hamster kidney monolayer cells (BHK 21 cl 13, Macpherson & Stoker, 1962).

A 1/4 dilution of each serum was prepared in the test medium and this was heated at 56° C. for 30 min. A twofold dilution series of this was then prepared in plastic micro-plates, using micro-diluter loops.* To each well containing the serum dilution was added an equal volume (50 μl.) of the virus suspension containing 100 TCID 50 virus. This was then incubated at 37° C. for 30 min. and then 25 μl. of cell suspension containing $2{\cdot}85 \times 10^6$ cells per ml. were added. The plates were covered with Sellotape and kept at 37° C. for 48–72 hr., by which time the test cell control wells had turned yellow. At this stage the Sellotape was removed and the medium in the wells allowed to become alkaline. When all the medium had become alkaline, the plates were again covered with Sellotape and replaced in the incubator. This procedure has the effect of removing any partial colour change that develops before any unneutralized virus is able to kill all the cells in the well. The plates were then read 24 hr. later and the titre expressed as the final dilution of serum neutralizing 100 TCID 50 virus.

The between-test standard deviation in this test when the virus input is between 1·5 and 2·5 TCID 50 was 0·35 ($\log_{10}$ reciprocal serum dilution). The regression coefficient for the correlation between virus input and serum antibody concentration was found to be 0·6.

RESULTS

The places at which samples were collected or from which slaughter cattle came immediately before sampling at the abattoir are shown in Fig. 1. The details of samples collected from cattle from the vaccinated area are shown in Table 1. Samples were deliberately collected from farms with no recent history of disease, as it was the presence of subclinical infection or symptomless 'carriers' unassociated with clinical disease that this study was concerned with. Even though the number of samples examined formed only a small proportion of the total cattle population, the proportion of virus 'carriers' found was still very low. Only six animals (0·49%) were found to be 'carriers' and of these four came from one farm in the same group of slaughter animals.

Table 2 shows the results of the samples collected in the non-vaccinated areas of the country. Here again, samples were only collected from herds with no recent history of disease. A higher proportion of 'carrier' animals (3·34%) was found. The infectivity titres of the positive samples were all less than $10^{1{\cdot}5}$ TCID 50/ml. of sample.

* Cooke Engineering Company, Virginia, U.S.A.

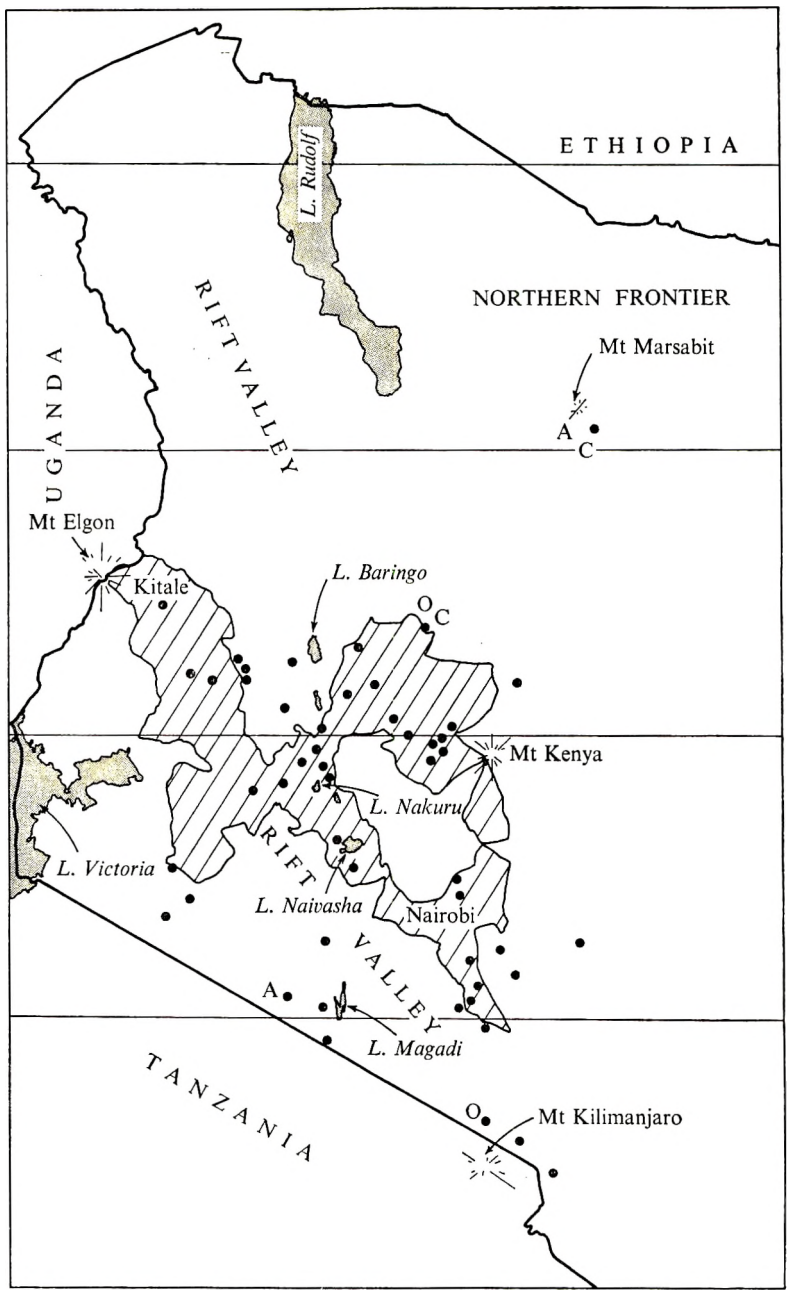

Fig. 1. Location of sampling points or sources of cattle sampled at the abattoir in carrier survey. The letters O, A and C indicate points at which carrier viruses of these types were isolated. ▨, Vaccinated area; ●, sampling points.

The immune status of the animals sampled from the vaccinated areas was also determined and the antibody concentrations to types O and A virus are shown in Table 3. The mean antibody concentrations, expressed as the logarithm of the reciprocal serum end-point dilution, were found to be 1·7 to type O and 1·6 to type A. To assess the significance of these antibody concentrations, sera from

Table 1. *Incidence of foot-and-mouth disease virus 'carrier' cattle in the vaccinated area*

No. of farms sampled	Total no. of samples	Average no. per farm	Total no. of samples as a % of cattle population in vaccinated area	Total no. of samples as a % of the no. of mature slaughter cattle	% of animals tested and found to be virus carriers
27	1231	46	0·017	1·0	0·49

Table 2. *Incidence of foot-and-mouth disease virus 'carrier' cattle in the non-vaccinated areas*

No. of cattle examined	No. examined as % of the cattle population in the areas sampled	% of animals tested and found to be virus 'carriers'
2219	0·09	3·34

Table 3. *Immune status of the herds sampled in the vaccinated area*

		Mean antibody concentrations ($\log_{10}$ reciprocal titres)		% of cattle with antibody concentrations of 1·35 or greater	
No. of farms sampled	No. of sera tested	Type O	Type A	Type O	Type A
27	1231	1·7	1·6	82	77

vaccinated cattle that had been challenged with 10^4 bovine ID50 of virus were assayed and the serum antibody concentration corresponding to a 50 % protection level was found to be 1·3. This concentration would probably correspond to a 100 % protection level in the field, where the challenge is likely to be much lower. Therefore, if all animals having a titre of 1·35 or greater are considered to be immune, 82 % of the sample would be immune to type O infection and 77 % to type A.

The duration of the 'carrier' state in the indigenous Boran cattle was also investigated by determining the incidence of 'carrier' animals in a group of 100 Boran steers 3½, 6½ and 15 months after clinical type C infection. The incidence at these times was, respectively, 8·5 %, 6·5 % and nil. The number of 'carriers' following type C infection therefore decreased steadily over a period of about a year.

The antigenic relation between two type O 'carrier' viruses, one isolated in the north of the country and the other in the south, and field viruses isolated during clinical outbreaks in these areas was examined by means of a micro cross-complement fixation test (Darbyshire, Hedger & Arrowsmith, 1972). The 'carrier' virus isolated in the north was antigenically dissimilar (R value = 19) to the field virus and could be classed as being a different subtype. This 'carrier' virus had been isolated from an animal that had no history of being associated with a clinical outbreak. The 'carrier' virus from the southern part of the country originated in a herd where there was serological evidence of type O infection within the previous

12 months. This isolate was still antigenically related (R value = 45) to the field outbreak strain from the area.

A type C 'carrier' virus isolated $6\frac{1}{2}$ months after the clinical outbreak was still antigenically similar to the field virus causing the outbreak (R value = 78).

A type A 'carrier' virus isolated in the south of the country two years after the last confirmed clinical outbreak was found to be antigenically related (R value = 53) to one field subtype strain found in that part of the country but dissimilar (R value = 34) to the other subtype strain.

DISCUSSION

The incidence of virus 'carrier' animals was found to fall steadily after a clinical outbreak of disease and few, if any, 'carriers' would be found 12 months later. This agrees with previous laboratory observations (van Bekkum *et al.* 1959; Burrows, 1966) and with field studies in Botswana (Hedger, 1970), where the incidence during an outbreak was 68 %. This had decreased to 38 % 6 months later and to 5·4 % 12 months later. The number of 'carriers' found in the enzootic non-vaccinated areas included in this study (3·34 %) was generally lower than that recorded in herds in Botswana (Hedger, 1968), where up to 20 % 'carriers' were found in three herds 7 months after a clinical outbreak and in one herd 12 months after an outbreak of the disease.

It has been demonstrated by Sutmöller, McVicar & Cottral (1968) and Hedger (1970) that vaccinated cattle may become 'carriers' when exposed to virus and that, if the challenge is low, this may occur without overt disease. It could be possible, in an enzootic area where the disease was controlled by vaccination, for subclinical infection to occur in partially immune animals and produce a proportion of 'carrier' animals. The results of this study show that, if total vaccination of the cattle population of the area is carried out and the spread of virus from clinical outbreaks is minimized by livestock movement control and quarantine procedures, then the establishment of the 'carrier' state through subclinical infection does not occur. As the number of clinical outbreaks decreases, so also does the number of virus 'carriers' until a situation is reached where the amount of virus in the environment is greatly reduced or the virus is even eliminated.

The results of the comparative studies on the antigenic relationship between 'carrier' viruses and field viruses indicate that there is an antigenic 'drift' away from the field viruses but these changes occur slowly. As yet there is no evidence that these new 'carrier' subtypes establish themselves in the area to cause clinical disease.

This study is published with the permission of the Director of Veterinary Services, Kenya.

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The foot-and-mouth disease virus subtype variants in Kenya

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SUMMARY

The subtype variants found in Kenya in the past ten years have been studied. The type O and type SAT 2 subtypes have a distinct geographical distribution which appears to be associated with livestock movement patterns. The type A viruses have a greater tendency to antigenic variation and their geographical distribution is less distinct. In type C only minor differences exist between the three viruses studied.

INTRODUCTION

Within each of the immunologically distinct foot-and-mouth disease virus type groups are found subtype variants which are distinguished by the fact that immunization to one subtype variant does not confer as solid an immunity to another variant of the same type as to the homologous strain (Traub & Möhlmann, 1946; Galloway, Henderson & Brooksby, 1948). These antigenic differences can be measured by serological methods and the variants classified into groups according to the degree of immunological variation exhibited (Davie, 1962, 1964). These subtype variants probably arise in the field through genetic change followed by selection in the host population, which may exhibit a range of susceptibility as a result of earlier infection or immunization. Pringle (1964) has shown that genetically stable mutants of subtype variants can be produced *in vitro* and that genetic recombination does occur in crosses of mutants of the same strain (Pringle & Slade, 1968; Pringle, Slade, Elworthy & O'Sullivan, 1970) and in crosses of serologically related strains (Pringle, 1965). It has also been demonstrated experimentally (Hyslop & Fagg, 1965; Fagg & Hyslop, 1966) that serial passage of a strain in partially immune cattle produces antigenic variation in that strain. The work described here was undertaken to determine the ecological pattern of the foot-and-mouth disease viruses present in Kenya as a contribution to the present and future control programme against the disease.

METHODS

Isolation of field viruses

Samples of vesicular epithelium were collected from the tongues of clinically infected cattle during the course of field outbreaks. Viruses from suspensions of such material were isolated on primary bovine thyroid cells (Snowdon, 1966) and typed by the micro complement-fixation test.

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Production of specific antisera

Each field virus was adapted to grow in either a baby hamster kidney cell line (BHK 21 Cl 13, Macpherson & Stoker, 1962) or in primary bovine kidney cells. The virus was then passaged once more in monolayers of the same cells in three Thomson bottles, each containing 220 ml. of maintenance medium consisting of Eagle's minimum essential medium plus 10% (v/v) tryptose phosphate broth. The virus was harvested 30–48 hr. later, when there was 100% cytopathic effect (CPE). This suspension was then centrifuged at 1600 *g* for 30 min. and the supernatant inactivated with 0·05% (v/v) acetylethyleneimine at 37° C. for 8 hr. and at 4° C. for a further 16 hr. The reaction was stopped with 2% (w/v) sodium thiosulphate. The inactivated antigen was then concentrated in a two-phase liquid polymer system (Albertsson, 1958) and purified by rate zonal centrifugation through a 25–45% sucrose gradient at 75000 *g* for $3\frac{1}{2}$ hr. The fraction containing the 140 S virion was identified by a micro complement-fixation test and emulsified in Freund's complete adjuvant. A 1 ml. dose of this vaccine was then given subcutaneously to an appropriate number of guinea-pigs of 700–800 g. body weight. One month later a second dose of freshly purified 140 S antigen, this time containing 0·25 mg. saponin as the adjuvant, was given. Ten days later the guinea-pigs were bled out and each serum assayed by a micro complement-fixation test. Antisera with antibody titres of 1/81 or greater were pooled and used in the subtyping test.

Identification of different subtypes

A micro complement-fixation test as described by Darbyshire, Hedger & Arrowsmith (1972) was used. Cross-fixation products were obtained for each group of viruses (Davie, 1964) and these are expressed as R values, where $R = 100\sqrt{(r_A r_B)}$ and

$$r_A = \frac{\text{complement fixed in reaction virus B + antiserum A}}{\text{complement fixed in reaction virus A + antiserum A}},$$

$$r_B = \frac{\text{complement fixed in reaction virus A + antiserum B}}{\text{complement fixed in reaction virus B + antiserum B}}.$$

In this study the following interpretation has been put on the R values obtained:

	R value
(i) Different subtype	40 or lower
(ii) Subtypes related but different	40–60
(iii) Same subtype	60 or greater

The figures applied to the significance of R values have beeen arbitrarily chosen in all the work on subtype strains. The figure of 70% was initially taken as it was believed that exposure following primary vaccination to a strain having an R value of less than this would result in significantly less protection than following exposure to the homologous strain. In the field work which is discussed in this paper we are dealing in general with a population of animals in which all but the youngest group have been revaccinated and, on the basis of observations on

vaccines in the field, we have chosen to classify only strains with an R value of 40 % or less as different. Those between 40 % and 60 % we have termed 'related' and over 60 % we have regarded as being within the limits of experimental error of the same subtype. It is to be expected that there will be a continuous series of variations and we therefore emphasize the arbitrary nature of the selection of values of R as having significance in cross-immunity studies.

The notation of viruses and subtype groups used

In this study three different notations are used to describe the virus strains. These are:

(*a*) The laboratory typing reference number of the strain, e.g. O–K120/64 is type O virus isolated in 1964.

(*b*) For reasons of clarity only, each isolate has been given a number, e.g. O-1, A-6, C-2, etc., and these are used in Fig. 1 to show the origin of these viruses.

(*c*) Where different subtype groups in Kenya have been established, these groups are referred to as O/K_1, O/K_2, O/K_3, etc. This should not be confused with the World Reference Laboratory (WRL), Pirbright, notations for different subtype groups which are O_1, O_2, O_3, etc. Although in this study no attempt was made to compare the different subtypes found with known subtype groups from other countries, some of the strains used have previously been classified by the WRL (J. B. Brooksby, personal communication), as follows:

subtype group O/K_1 = WRL group O_9,
subtype group A/K_2 = WRL group A_{23},
subtype group SAT $2/K_2$ = WRL group SAT 2_3.

Virus strain A-K140/69, which is related to subtype groups A/K_3, A/K_4 and A/K_6, has also been shown to be related to WRL group A_{22}.

RESULTS

Field outbreaks are mainly caused by types O and A viruses. Type SAT 2 outbreaks do occur but are less common. In 1970 51 % of all outbreaks were type O, 30 % were type A and 17 % were type SAT 2. In 1971 66 % were type O, 19 % were type A and 14 % were type SAT 2. Type C outbreaks occur, but very infrequently. In this study, virus isolates of types O, A, C and SAT 2 from field outbreaks in different parts of the country were compared (Fig. 1). The antigenic interrelations of each type group of viruses, expressed as R values, are given in Tables 1–4.

In order to try to relate the R values obtained with possible significance to vaccination, it was first necessary to show that the complement-fixation test used was not influenced to any degree by the presence of non-specific antibodies in the antisera to the 12 S protein subunits present in most unpurified virus suspensions. These were shown by Bradish & Brooksby (1960) to fix complement with heterotypic antisera and could therefore be responsible for increased values of R. Real differences between the entire virion which is the immunogenic particle (Randrup, 1954; Brown & Crick, 1959) would then be less apparent. Consequently, a series of complement-fixation tests was carried out with three type O subtype variants

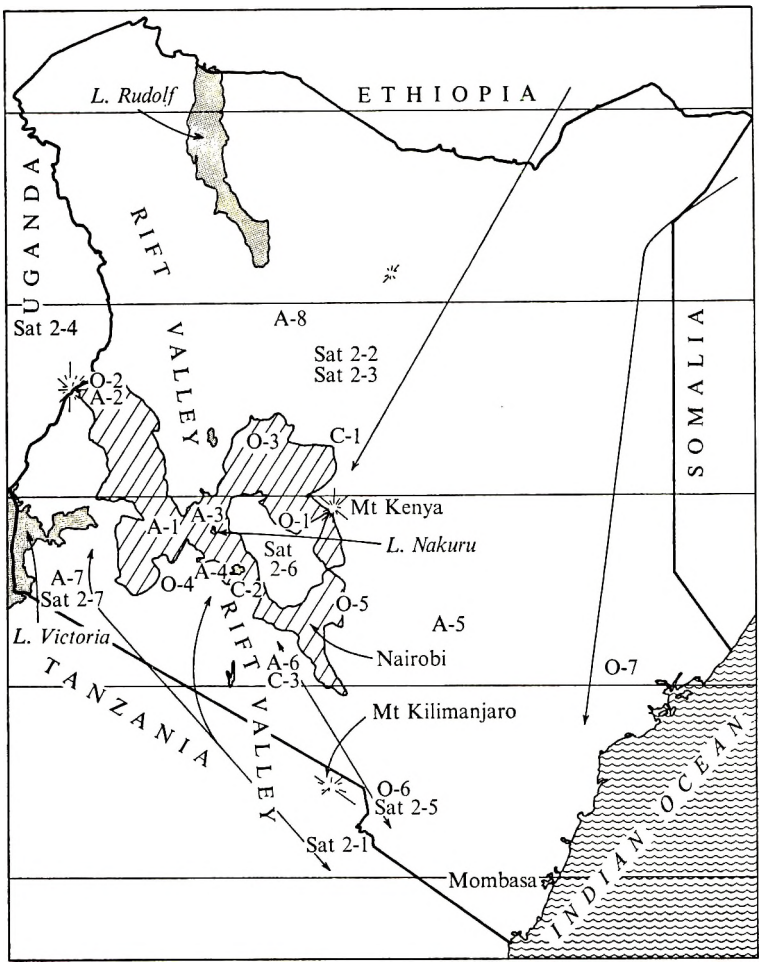

Fig. 1. Location of field outbreaks from which the various subtypes were originally recovered. ▨, Vaccinated area. →, Livestock movements.

Table 1. R *values – type O viruses*

Virus	Laboratory typing reference no.	Antiserum						
		O-1	O-2	O-3	O-4	O-5	O-6	O-7
O-1	K120/64	100	—	—	—	—	—	—
O-2	K248/71	47	100	—	—	—	—	—
O-3	K1/72	65	45	100	—	—	—	—
O-4	K171/72	36	35	25	100	—	—	—
O-5	K61/71	38	16	—	23	100	—	—
O-6	K4/71	21	14	—	39	87	100	—
O-7	K217/72	78	40	—	34	37	21	100

and three type A variants, using both unpurified virus harvests and preparations of the purified 140 S particle. The *R* values obtained are compared in Tables 5 and 6 and are essentially the same for both antigens, indicating that in routine tests using antisera raised by the method described the presence of 12 S antigen is relatively unimportant.

Table 2. R *values – type A viruses*

Virus	Laboratory typing reference no.	Antiserum A-1	A-2	A-3	A-4	A-5	A-6	A-7	A-8
A-1	K18/66	100	—	—	—	—	—	—	—
A-2	K46/65	27	100	—	—	—	—	—	—
A-3	K140/69	36	43	100	—	—	—	—	—
A-4	K180/71	15	36	60	100	—	—	—	—
A-5	K104/72	15	34	40	32	100	—	—	—
A-6	K179/71	12	31	63	100	44	100	—	—
A-7	K27/72	17	25	39	—	24	24	100	—
A-8	K82/72	21	30	68	—	23	25	37	100

Table 3. R *values – type C viruses*

Virus	Laboratory typing reference no.	Antiserum C-1	C-2	C-3
C-1	K267/67	100	—	—
C-2	K182/69	68	100	—
C-3	K42/73	52	—	100

Table 4. R *values – type SAT 2 viruses*

Virus	Laboratory typing reference no.	Antiserum SAT 2-1	SAT 2-2	SAT 2-3	SAT 2-4	SAT 2-5	SAT 2-6	SAT 2-7
SAT 2-1	T5/68	100	—	—	—	—	—	—
SAT 2-2	K3/57	18	100	—	—	—	—	—
SAT 2-3	K159/70	46	26	100	—	—	—	—
SAT 2-4	U6/70	8	14	—	100	—	—	—
SAT 2-5	K83/72	61	22	23	13	100	—	—
SAT 2-6	K147/72	83	20	32	18	43	100	—
SAT 2-7	K162/72	49	21	32	—	32	50	100

Table 5. *A comparison of* R *values obtained using purified and unpurified antigen – type O viruses*

Serum	O-1 Purified antigen	O-1 Unpurified antigen	O-4 Purified antigen	O-4 Unpurified antigen	O-6 Purified antigen	O-6 Unpurified antigen
O-1	100	100	—	—	—	—
O-4	44	36	100	100	—	—
O-6	32	21	39	39	100	100

Type O viruses

The *R* values of the type O viruses included in this study are given in Table 1. These may be placed in three subtype groups, as shown in Table 7.

The viruses of group O/K_1 all originated in the north and east of the country, while those of groups O/K_2 and O/K_3 were isolated in the south. The livestock movement patterns that traditionally occur within these two regions are shown

Table 6. *A comparison of* R *values obtained using purified and unpurified antigen – type A viruses*

	A-4		A-1		A-5	
Serum	Purified antigen	Unpurified antigen	Purified antigen	Unpurified antigen	Purified antigen	Unpurified antigen
A-4	100	100	—	—	—	—
A-1	6	15	100	100	—	—
A-5	36	32	12	22	100	100

Table 7. *Subtype groups – type O viruses*

Group	Virus	Related virus
O/K_1	O-1, O-3, O-7	O-2
O/K_2	O-4	—
O/K_3	O-5, O-6	—

Table 8. *Subtype groups – type A viruses*

Group	Virus	Related virus
A/K_1	A-1	—
A/K_2	A-2	—
A/K_3	A-4, A-6	A-3
A/K_4	A-5	A-3, A-6
A/K_5	A-7	—
A/K_6	A-8	A-3

Table 9. *Subtype groups – type SAT 2 viruses*

Group	Virus	Related viruses
SAT $2/K_1$	SAT 2-1	SAT 2-3
	SAT 2-5	SAT 2-7
	SAT 2-6	—
SAT $2/K_2$	SAT 2-2	—

in Fig. 1. From the north cattle move south into the Rift Valley area and coastal belt. In the south-west livestock movement is extensive but mainly confined to this region, although there is some movement into the Rift Valley and the area bordering Lake Victoria. Of the two strains isolated in the Rift Valley region, strain O-2 from the north-west is antigenically related to the northern subtype while strain O-5 is the same subtype as one of the southern strains (O-6).

Type A viruses

Amongst the type A viruses included in this study (Table 2), six subtype groups have emerged as shown in Table 8. The geographical distribution of the different type A subtypes does not show such a distinct regionalization as the type O subtypes, although each subtype group does originate in a different part of the country. The larger proportion of distinct groups found indicates a greater tendency to antigenic variation in the type A viruses. Strain A-3 is antigenically related to groups A/K_3, A/K_4 and A/K_6.

Type C viruses

Few outbreaks of type C infection have occurred and only three strains have been examined. These were all closely related (Table 3) but from the few data available it cannot be concluded that C strains are more antigenically stable.

Type SAT 2 viruses

The SAT 2 viruses examined (Table 4) fell into two subtype groups as shown in Table 9. One subtype group contains those viruses isolated in the south of the country, while the other contains the northern strain SAT 2-2. Strain SAT 2-3 isolated in 1970 has some antigenic relation to one of the southern strains, SAT 2-1, but not to the others. It is, however, a different subtype from strain SAT 2-2 isolated at the same place in 1957. The geographical distribution of the type SAT 2 subtypes follows the same pattern as the type O subtypes.

DISCUSSION

In an enzootic area subtype variants may arise by mutation of the parent strain or by recombination between subtype variants where a dual infection occurs. Any mutation of the genome affecting the antigenic composition of the surface proteins of the virion will be immediately expressed and such a variant will then be subject to natural selection in the population of immune and partially immune animals (Pringle, 1969). Although such selection pressure itself may act towards the appearance of subtype variants, these variants will not easily establish themselves in this population. New subtypes are only likely to arise by gradual transition from the parent strain to establish themselves as stable mutants (Pringle, 1964; Hyslop & Fagg, 1965). The results of this study indicate that this is probably what happens in an enzootic area. Relatively few different subtypes were found, while the number of serologically related but still antigenically dissimilar strains found, particularly amongst the type A and type O viruses, supports the view that there is a gradual transition away from the parent strains. Where different subtypes were found, they were usually from different geographical areas. There appeared to be some relation between the distribution of the different subtypes and livestock movement patterns (Fig. 1). In general, the strains found in the north of the country are different from those found in the south and west. There is little direct movement of livestock between these areas; rather, livestock movement takes place from these areas towards the Rift Valley and Central Highlands.

The significance of the presence of different subtypes in relation to disease control by regular vaccination is not clear. In Kenya, since 1968 a vaccination programme requiring routine vaccination of all cattle over three months of age against types O and A every 6 months has been carried out in the Central Highlands (Fig. 1). This programme was started in a small area and gradually extended so that by 1973 the area shown in Fig. 1 was being covered. An inactivated vaccine containing strains of subtype groups A/K_1 and O/K_1 was used. Many of the field strains, particularly those of type A, show quite marked antigenic differences from the vaccine strains yet the number of type O and A outbreaks of disease in the vaccinated areas has fallen from 40 in 1968 to nine in 1969, five in 1970, two in

1971, three in 1972 and four in 1973. These results are largely due to a good level of herd immunity being maintained and, at the same time, minimizing the challenge through rigid quarantine procedures following a disease outbreak. Whether the same results would have been achieved if, following the appearance of a new subtype, no quarantine measures were enforced and the weight of challenge allowed to build up is uncertain.

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The isolation of *Escherichia coli* from a poultry packing station and an abattoir

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SUMMARY

The distribution and serotype of strains of *Escherichia coli* from a poultry packing station and an abattoir are described. The results indicated that animal faecal strains contaminated the environment and the animal carcasses.

Using 150 O antisera, a high proportion of the *E. coli* strains were non-typable. This suggests that the serotype distribution of *E. coli* in animals is different from that in man.

Strains with single antigenic differences were isolated, and the possibility of genetic transfer of these antigenic structures is suggested.

INTRODUCTION

We have previously briefly described the isolation of *Escherichia coli* from a poultry packing station and an abattoir (Shooter, Cooke, Rousseau & Breaden, 1970). We now report the distribution and serotypes of *E. coli* from these sources and provide further evidence about the spread of *E. coli* in these environments.

MATERIALS AND METHODS

The poultry packing station and the abattoir have been described (Shooter *et al.* 1970). Both were well maintained and the standards of hygiene were good.

Swabs were taken from the interior and exterior of the chicken carcasses; cloacal swabs were taken after killing but before processing. Giblets were cut up and suspended in $\frac{1}{4}$ strength Ringer's solution for examination. Swabs were taken from the environment of the poultry packing station and the water in the de-feathering and cooling tanks was also sampled.

In the abattoir swabs were taken from the skin and flesh of the animals. Rectal swabs were taken after the animals were killed but before evisceration. Swabs were also taken from the environment of the abattoir.

The O and H antigens of strains of *E. coli* were identified using 150 O antisera and 50 H antisera. The methods used were based on those described by Bettelheim & Taylor (1969).

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Table 1. Escherichia coli *types found in a poultry packing station and an abattoir*

	No. of strains		
	Poultry	Pigs	Beef
O typable	149	24	116
O non-typable	135	46	183
Rough	65	22	58
H typable	271	68	344
H non-typable	15	11	4
Non-motile	63	13	9
Total	349	92	357

RESULTS

Poultry packing station

Two surveys of the poultry packing station were carried out, in which 349 strains of *E. coli* were isolated. These were from the defeathering and cooling tanks and from the carcasses at all stages of processing. The O and H typing characteristics of these strains are shown in Table 1.

Many O groups were found to be associated with a number of H antigens and to have different antibiotic sensitivity patterns; for example, O69.H38 and O69.H48 resistant to tetracycline and streptomycin were isolated and O69.H38 resistant to tetracycline. In the second survey similar results were obtained except that different serotypes were isolated.

Abattoir

The contamination of the environment was much less than that of the poultry packing station. A total of 357 strains of *E. coli* were isolated during studies when cattle were being slaughtered and 92 when pigs were being slaughtered. The number of these which were typable is shown in Table 1.

The serotypes commonly isolated from cattle were O17.H18, O58.H40, O91.H7, O113.H4, O132.H28, O146.H21 and O non-typable with H antigens 2, 7, 8, 10, 19, 21 and 31 and rough strains with H antigens 19 and 42. Many of these were also recovered from the environment or from the carcasses.

From the pigs also there was a wide scatter of serotypes. Those of which more than one was isolated were O68.H12, O97.H16, O103.H43 and O non-typable with H antigens 8 and 10 and rough strains with H antigens 16 and 30.

DISCUSSION

Strains of *E. coli* are frequently isolated from food and there is evidence to suggest that these strains subsequently form part of the human faecal population of *E. coli* (Shooter *et al.* 1970). The present study is an attempt to determine the sources of *E. coli* on meat and we have studied the isolation of *E. coli* during the procedures followed at a chicken packing station and an abattoir.

One of the most interesting results to come out of this investigation was that even though a nearly complete set of *E. coli* O antisera was used only 289 out of

798 strains could be serotyped. During a study on human faeces carried out at the same time (Bettelheim, Faiers & Shooter, 1972) only two out of 1580 strains could not be identified with 150 O sera. This seems to indicate that strains of animal origin may comprise O groups which are not commonly encountered in human faeces and have therefore not been given O numbers. It is unlikely that only a few such O groups occur because in the present study 13 different H antigens were associated with these unknown *E. coli* O serotypes.

In the present study it could be shown that a strain of *E. coli* O69.H38 which was resistant to both streptomycin and tetracycline and which was first isolated from the rectal swab of a chicken could be isolated throughout the process and it was present in the final prepared carcasses. The presence of 4 markers on this strain marked it out very easily from all other strains. It was nevertheless interesting to note that a number of strains were present in the process carrying only some of these markers. These included strains of *E. coli* O69.H38 which were resistant only to tetracycline, and strains resistant to both antibiotics but with single differences in antigenic structures; O69.H48; O51.H38; and O N.T.H38. Whether these antigenic determinants can be transferred in the same way as the resistance to antibiotics is not known although this appears to be a possibility. Antigenic variation has also been observed among the *E. coli* isolated from chronic urinary tract infections (Bettelheim & Taylor, 1969).

A sensitive strain of *E. coli* O25.H45 which was first isolated from the feather softening bath could also be isolated from most of the subsequent stages to the prepared carcasses. It is interesting to note that the sensitive strain that was found was probably present in the water, while an antibiotic-resistant strain of the same serotype was first observed in the rectal swab of a chicken.

The results in both the abattoir and the poultry packing station indicate that there is transfer of strains from the faeces of the animals to the environment and that the strains of *E. coli* found on the carcasses of poultry, cattle and beef will originate from the faeces of the animal and from the environment and will reflect the history of the carcass.

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Pseudomonas aeruginosa and the general hospital: a six-year survey

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SUMMARY

The incidence of infections caused by *Pseudomonas aeruginosa* did not increase significantly among general surgical and medical patients between 1967 and 1972, and the majority of such infections were trivial. Serious infections were virtually confined to the intensive care unit and the renal transplant unit, and were usually associated with major trauma, surgical mishap or immunosuppression. The majority of these patients had received prior antibiotic therapy. Persistent isolation of *Ps. aeruginosa* from surgical wounds was often associated with severe intra-abdominal sepsis, and antibacterial therapy was commonly ineffective in these cases. Apparently susceptible patients did not necessarily acquire infection, though the organism was present in their environment. It is suggested that this may reflect a variation of virulence among environmental strains of *Ps. aeruginosa*, and that further study of this aspect may contribute to improved control of infection.

INTRODUCTION

In recent years, some hospitals have reported an increased incidence of Gram-negative bacilli in clinical material. Members of the Enterobacteriaceae have predominated (Dans, Barrett, Casey & Finland, 1970; Adler, Burke & Finland, 1971) but *Pseudomonas aeruginosa* has accounted for significant numbers of isolations in certain instances (Harris, Orwin, Colquhoun & Schroeder, 1969; Noone & Shafi, 1973). To some extent, the frequency with which this species is isolated reflects the type of hospital and the opportunities available within it for cross-infection. Many reports deal with epidemics of infection which have occurred in specialized units where basic hygienic precautions have been neglected.

Although isolation of *Ps. aeruginosa* from specimens is not necessarily synonymous with infection, serious disease such as septicaemia and necrotizing pneumonia occasionally occurs in patients with underlying immunological deficiencies. However, the incidence of severe *Ps. aeruginosa* infection among the general hospital in-patient population remains ill-defined. In an attempt to identify and assess the relative importance of factors predisposing to major infection, a survey has been made of all isolations of the species from in-patients of this hospital over the years 1967–72.

METHODS

The hospital has 329 beds. It undertakes acute general medicine and surgery, and also has wards for orthopaedics, urology, ophthalmic surgery and ear, nose and throat surgery. There is a five-bedded intensive care unit (I.C.U.), previously described in detail (Harris *et al.* 1969), and a four-bedded renal transplant unit (R.T.U.). Frequent (often daily) bacteriological monitoring is carried out on relevant clinical material from patients in these two units. Bacteriological investigation of patients in other wards is more variable, but on the whole the laboratory is well used, the total number of specimens investigated annually having risen from 19073 in 1967 to 23836 in 1972.

Most strains of *Ps. aeruginosa* were identified on the basis of their characteristic colonial appearance. Less typical strains were investigated further. Isolations were classified as 'transitory' (isolation on one occasion only) or 'persistent' (isolation on two or more occasions from the same patient). A few strains were pyocine-typed by the method of Gillies & Govan (1966). The term 'strain' is used to indicate an organism isolated from a particular patient, irrespective of the number of occasions on which it was isolated. Relevant additional information was obtained from the clinical records, and the necropsy report was consulted in the case of patients who died.

RESULTS

Of a total of 73725 patients admitted to the hospital, *Ps. aeruginosa* was isolated from the wounds, urine or respiratory tracts of 388; the distribution of these strains is shown in Table 1.

Although 73 % of the isolations were transitory, persistent isolation was as common as, or even commoner than, transitory isolation in patients from the two highly specialized units. A quarter of the strains were first isolated on the I.C.U. or R.T.U. Of the remaining 280 strains, 224 were distributed over nine general medical and surgical wards and 56 were isolated from patients in the ophthalmic, orthopaedic and ear, nose and throat (E.N.T.) units. The interval elapsing between successive isolations varied widely for different types of ward. For example, in the medical wards the mean interval was 148 days, in the general surgical wards it was 71 days, and in the urology ward it was 25 days. Despite the greater susceptibility of its patients, the mean interval between isolations on the renal transplant unit was 95 days, which was probably attributable to the existence of individual cubicles and consequent lack of opportunity for cross-infection. The I.C.U., which has only one cubicle, was the only unit where 'clustering' of infection was noted (in the sense of three or more infections occurring within a fortnight). This happened on three occasions, the first episode involving six patients and the others each involving three. However the mean interval between isolation of successive strains on this unit was 21 days; on twelve occasions there were periods of 6 weeks when no patient in the unit was colonized by *Ps. aeruginosa*, and the longest interval recorded was 17 weeks.

Table 2 shows the annual incidence of *Ps. aeruginosa* infection in relation to

Table 1. *Number of patients from whom* Pseudomonas aeruginosa *was isolated*

Initial isolation:		Location of patient when first isolated: Surgical wards	Medical wards	I.C.U.	R.T.U.
Respiratory tract	*a*	15	21	27	1
	b	0	7	53	0
Surgical wounds, chronic discharges	*a*	86	20	11	0
	b	11	0	6	3
Urine	*a*	85	25	3	0
	b	8	2	2	2

a, Isolated on one occasion only.
b, Isolated on two or more occasions.

Table 2. *Number of patients infected annually by* Ps. aeruginosa *and* Staph. aureus

Year	Admissions	No. infected: *Ps. aeruginosa*	*Staph. aureus*	*Ps. aeruginosa*:*Staph. aureus* ratio: Whole hospital	I.C.U.
1967	11441	67 (0·58)*	201 (1·75)	0·33	0·83
1968	11516	76 (0·67)	242 (2·10)	0·31	0·91
1969	11813	65 (0·55)	290 (2·45)	0·22	0·48
1970	12649	56 (0·44)	314 (2·47)	0·18	0·50
1971	13068	74 (0·56)	308 (2·48)	0·24	0·94
1972	13238	50 (0·38)	388 (2·93)	0·13	0·93
Total	73725	388 (0·52)	1743 (2·36)	0·22	0·71

* Figures in parentheses indicate infections expressed as percentages of annual admissions.

annual admissions and to the number of strains of *Staph. aureus* isolated during the same period.

The proportion of patients infected by each species showed little variation over the years. However the ratio of *Ps. aeruginosa* to *Staph. aureus* infections was consistently greater in the I.C.U. than in the hospital as a whole. Thirty-four (33 %) of those patients infected by *Ps. aeruginosa* in the I.C.U. also had either previous or concurrent staphylococcal infection, as compared with 19 (6·7 %) of those acquiring *Ps. aeruginosa* in the general wards.

Thirty-seven patients acquired respiratory tract colonization by *Ps. aeruginosa* during assisted ventilation, 27 (73 %) of them having been previously infected by other bacteria (Table 3). No patient was colonized by *Ps. aeruginosa* in the first 3 days of ventilation, although 20 strains (54 %) were isolated during the first week. However, prolonged ventilation did not necessarily lead to acquisition of *Pseudomonas*; of 21 other patients who were ventilated for 7 days or longer, seven remained free of any bacterial infection, six acquired *Staph. aureus*, seven coliform bacilli and one *Haemophilus influenzae*. These patients were broadly comparable in age and severity of illness to those infected by *Ps. aeruginosa* and, since there was a high incidence of multiple antibiotic-resistance in the coliform bacilli and

Table 3. *Colonization of tracheostomies in relation to duration of intermittent positive pressure ventilation (I.P.P.V.) in patients who eventually acquired* Ps. aeruginosa

	Days after the institution of I.P.P.V.															
No. colonized by ...	1	2	3	4	5	6	7	8	9	10	11	12	14	15	21	26	28
H. influenzae	.	1	1	.	.	.	.	.	.	.	.	1	.	.	.	.	.
β-Haemolytic Streptococcus	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.
Coliform bacilli	3	1	1	.	1	1	3	.	.	.	2	.	1	1	.	.	.
Proteus	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.
Staph. aureus	3	.	1	1	1	1	.	.	.	.	.	.	.	.	1	1	.
Ps. aeruginosa	.	.	.	5	5	4	6	2	1	1	3	2	3	3	.	1	1

staphylococci isolated from both groups, predisposing antibacterial therapy was essentially similar.

Septicaemia was suspected in 26 patients (all from either the R.T.U. or I.C.U.). Blood from these patients was cultured on 39 occasions, but *Ps. aeruginosa* was isolated in only one instance (from a severely burned patient). Only five of these patients were receiving antibiotics capable of inhibiting the growth of *Ps. aeruginosa* at the time of blood culture.

The 48 patients who died were between 6 and 86 years old; 24 were below 60 years of age, and only three were over seventy. In all cases, *Ps. aeruginosa* was only one of several bacterial species which had infected the patient during life. Nine of the patients had multiple injuries, three had received renal transplants, 15 had required intensive care after other forms of surgery (mostly abdominal) and 15 had been ventilated for respiratory failure of varying aetiology. The remainder suffered from intracranial haemorrhage or encephalitis (4), severe burns (1) and acute renal failure following septicaemia (1). Factors contributing to infection included corticosteroid therapy and immunosuppression (3), diabetes mellitus (1) and malignancy (2). Necropsy was performed on 27 patients. It revealed a high incidence of major intra-abdominal sepsis in the surgical cases, eight patients having generalized peritonitis and a further five subphrenic or retroperitoneal abscesses. Bronchopneumonia was present in 14 patients, but the necrotizing vasculitis which has been recorded as characteristic of *Pseudomonas* infection was not encountered. Post-mortem bacteriology usually yielded a mixed flora similar to that isolated in life.

DISCUSSION

The epidemiology and pathogenesis of *Ps. aeruginosa* infections present a complex problem. The species can survive in moist environments on a minimum of nutrient, grow in dilute antiseptics and resist the action of many antibiotics. It is widely disseminated through most hospitals yet infection remains uncommon and life-threatening infection rare. The present survey does not suggest a significant increase in infections over the last 6 years, and the pattern remains that of trivial infection or transient colonization in most cases, with occasional episodes of more severe infection in seriously ill patients. It is difficult to compare hospitals, since

institutions vary in methods of therapy and cross-infection control. However, the situation in Sheffield is essentially similar to that reported for Edinburgh during 1961 and 1962 (Gould, 1963). More recently, Noone & Shafi (1973) reported an annual infection rate of 1·4–1·8 % of admissions to the Central Middlesex Hospital between 1968 and 1970; a vigorous cross-infection control policy led to an incidence of 0·6 % in 1971. All three surveys suggest that, although control measures may effectively reduce the number of trivial infections and transient colonizations, there remains a small group of susceptible patients in whom it is extremely difficult to prevent infection by *Ps. aeruginosa*. Like Gould, we did not find the extremes of age excessively represented in our cases, and comparatively few had an identifiable immunological deficiency. The chief predisposing factor appeared to be tissue damage, either due to accidental trauma or to radical surgery. In this context, surgical mishap (e.g. breakdown of anastomoses) appeared to be significant.

The intervals elapsing between successive isolations suggested that direct spread of infection between patients was unimportant on the medical and surgical wards. More opportunities exist for cross-infection on an intensive care unit, and it is difficult to explain the failure of some patients to acquire infection with *Ps. aeruginosa* when there were already infected patients in the unit. This may represent the success of aseptic nursing techniques, but it may also reflect subtle differences in the susceptibility of the patients. Although several toxins have been extracted from *Ps. aeruginosa*, the contribution of each fraction to the pathogenicity of the organism is uncertain, and Liu (1964) has suggested that the amount of individual toxins produced is dependent on the nature of the biochemical abnormalities present in damaged tissues. Such biochemical differences may be influential in determining whether *Ps. aeruginosa* is able to establish itself in the tissues of various patients who otherwise appear equally susceptible.

Opinions differ on the importance of environmental colonization. Some workers consider that the strains of *Ps. aeruginosa* endemic in sinks and other moist situations constitute a major source of infection (Teres *et al.* 1973). Others have failed to find such an association (Lowbury *et al.* 1970). However, the possession of toxic factors by strains unassociated with infection has not been investigated on a large scale, and further work is required to clarify the situation. The limited amount of pyocine typing performed on our strains revealed poor correlation between strains isolated from patients and from their immediate environment.

A striking finding in this survey was the association of persistent isolation of *Ps. aeruginosa* from surgical wounds with a subsequent finding of serious intra-abdominal sepsis. This is of some practical importance, since antibacterial therapy directed towards *Pseudomonas* isolated from a wound is unlikely to benefit the patient who has a subphrenic abscess requiring drainage.

Many of the seriously ill patients had received antibiotic therapy for other infections before the isolation of *Pseudomonas*, and it could be maintained that a more restricted use of antibiotics would reduce the incidence of *Ps. aeruginosa* infection. Price & Sleigh (1970) and Klastersky, Beuner & Daneau (1971) have shown that patients with *Klebsiella* or *Staph. aureus* infection of the respiratory tract can be treated successfully with vigorous physiotherapy and scrupulous

bronchial toilet alone. However there will probably always be an irreducible minimum of patients for whom antibacterial therapy is indicated. These will inevitably be the most gravely ill cases, and most of them will be receiving sophisticated medical care at some stage. Experience in the intensive care unit of this hospital showed that 84·2 % of strains of *Staph. aureus* isolated from patients in the unit were already present at the time of admission, and that the problem of infection was therefore related to conditions in the wards from which the patients had been transferred (Harris, 1973). This did not apply in the case of *Ps. aeruginosa*, since virtually all the seriously ill patients who acquired the organism did so while in the unit. The inherent virulence of strains endemic in the environment is therefore crucial, and an intensive study of the pathogenicity of such strains is required.

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Enterotoxin production by strains of *Staphylococcus aureus* isolated from foods and human beings

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SUMMARY

Enterotoxin production by strains of *Staphylococcus aureus* isolated from routine samples of foods and from human beings was investigated. Twenty-one to 26 % of 112 strains isolated from raw meat, sausages and poultry and 32–36 % of 183 strains isolated from cooked foods, e.g. meat, chicken and frozen seafoods, produced enterotoxins A, B, C, D or E. *Staph. aureus* isolated from raw meat and chicken less frequently produced enterotoxins A, B, C or E and more frequently enterotoxin D, than those from cooked meat and seafoods. Of the 113 strains isolated from cheese and raw milk 6–11 % produced enterotoxin and most of these produced enterotoxin D. Only a few strains isolated from foods produced enterotoxin E. Results of enterotoxin tests on *Staph. aureus* from human beings resembled those on strains from cooked foods.

INTRODUCTION

The cause of staphylococcal food poisoning is the enterotoxin produced by a strain of *Staphylococcus aureus* in the implicated food. So far five enterotoxins have been identified, designated A, B, C, D and E. Staphylococci which produce enterotoxins A, or A and D together, have been found in 69–75 % of reported outbreaks of staphylococcal food poisoning in the U.S.A. and the U.K. Strains which produce enterotoxin D alone or together with enterotoxin C have been found in about 10 % of the outbreaks and those which produce enterotoxin B are only rarely found (Casman, Bennett, Dorsey & Issa, 1967; Gilbert & Wieneke, 1973).

The purpose of this investigation was to study the distribution of enterotoxin production among strains of *Staph. aureus* isolated from samples of food unconnected with outbreaks of food poisoning, from admission swabs of patients who entered hospital and from lesions of hospital patients.

MATERIALS AND METHODS

Strains of *Staph. aureus* were isolated from food samples during routine bacteriological examinations at the Food Hygiene Laboratory, Colindale, and at several other Public Health Laboratories. A few strains were isolated by direct culture on phenolphthalein diphosphate agar that contained polymyxin* (PPAP) (Hobbs,

* In the examination of cheese polymyxin was omitted from the medium.

Table 1. *Enterotoxin production by strains of* Staphylococcus aureus *isolated from various sources: % of strains which produce enterotoxin A, B, C, D or E*

Strains isolated from	No. of strains tested	No. of strains which produce entero-toxin A–E	% of strains which produce entero-toxin A–E	% of strains which produce enterotoxin				
				A	B	C	D	E
Frozen raw chicken	50	13	26	—	2·0	6·0	22	—
Raw meat and sausages	62	13	21	1·6	—	1·6	19	—
Cooked chicken	53	17	32	7·6	1·9	17	19	1·9
Cooked meat	50	18	36	10	8·0	14	10	2·0
Frozen cooked prawns, lobster, crab and crayfish	80	28	35	17	8·7	17	3·7	1·2
Salami and pâté	16	3	19	13	—	6·3	—	6·3
Cream and artificial cream mostly in pastries	28	9	32	7·2	7·2	11	11	3·6
Raw milk	50	3	6	—	—	—	6	—
Cheese	63	7	11	1·6	1·6	1·6	9·5	—
Hospital admission swabs	101	38	38	16	7·0	11	7·0	2·0
Lesions among hospital patients	199	91	45	20	14	16	9·5	2·0
Food poisoning out-breaks*	120	113	94	73	1·7	15	40	2·5

* A representative strain from each of 120 separate outbreaks was selected.

Kendall & Gilbert, 1968), but most were isolated through enrichment culture (cooked meat medium that contained a total of 10 % salt) and subculture on PPAP. Phosphatase-positive colonies were tested for coagulase production in 10 % human plasma broth. Coagulase-positive strains were incubated in a sac-culture flask and the culture filtrates were tested for the presence of enterotoxin with the use of the double gel diffusion slide test (Šimkovičová & Gilbert, 1971). Strains of *Staph. aureus* isolated from hospital admission swabs and from lesions of hospital patients were made available to us by Dr M. T. Parker and Mr J. H. Hewitt of the Cross-Infection Reference Laboratory at Colindale.

The culture filtrates were tested immediately after their preparation for the presence of enterotoxins A, B and C. They were then stored at 4° C. and tested for the presence of enterotoxins D and E after a maximum period of 4 years. Enterotoxins A, B and C could still be demonstrated in filtrates stored for 4 years and several of the early filtrates were positive for enterotoxins D and E.

All strains were phage-typed by the Cross-Infection Reference Laboratory at Colindale; the international basic set of 22 phages was used.

RESULTS

Tables 1 and 2 give the results of enterotoxin tests on strains of *Staph. aureus* isolated from routine samples of foods, from swabs taken from patients at the time of their admission into hospital and from lesions of hospital patients. For com-

Table 2. *Enterotoxin production by strains of* Staphylococcus aureus *isolated from various sources: number of strains which produce one or more enterotoxins*

Strains isolated from	No. of strains which produce enterotoxin A–E	No. of strains which produce enterotoxin												
		A	B	C	D	E	AB	AC	AD	BD	CD	CE	ACD	ACE
Frozen raw chicken	13	—	1	1	9	—	—	—	—	—	2	—	—	—
Raw meat and sausages	13	1	—	—	11	—	—	—	—	—	1	—	—	—
Cooked chicken	17	—	1	4	3	1	—	1	3	—	4	—	—	—
Cooked meat	18	3	4	5	1	1	—	—	2	—	2	—	—	—
Frozen cooked prawns, lobster, crab and crayfish	28	6	6	7	—	—	1	4	1	—	1	—	1	1
Salami and pâté	3	2	—	—	—	—	—	—	—	—	—	1	—	—
Cream and artificial cream mostly in pastries	9	2	2	1	2	—	—	—	—	—	1	1	—	—
Raw milk	3	—	—	—	3	—	—	—	—	—	—	—	—	—
Cheese	7	—	—	—	5	—	—	1	—	1	—	—	—	—
Hospital admission swabs	38	12	5	9	5	2	2	1	1	—	1	—	—	—
Lesions among hospital patients	91	12	12	26	6	4	15	3	9	1	2	—	1	—
Food poisoning outbreaks*	113	53	—	4	8	2	2	3	30	—	10	1	—	—

* A representative strain from each of 120 separate outbreaks was selected.

Table 3. *Enterotoxin production by strains of* Staphylococcus aureus *of different phage-group isolated from routine foods*

Phage-group	No. of strains tested	No. of strains which produce enterotoxin A–E	% of strains which produce enterotoxin A–E	No. of strains which produce enterotoxin												
				A	B	C	D	E	AB	AC	AD	BD	CD	CE	ACD	ACE
I	65	15	23	2	2	4	3	1	—	—	—	—	3	—	—	—
II	28	3	11	—	2	1	—	—	—	—	—	—	—	—	—	—
III	145	42	29	7	2	2	21	—	—	2	3	1	4	—	—	—
IV	18	2	11	—	—	—	2	—	—	—	—	—	—	—	—	—
I/III	81	20	25	4	2	5	3	—	1	1	3	—	1	—	—	—
II/III I/II/III	7	3		—	1	—	—	—	—	—	—	—	1	—	1	—
I/IV III/IV I/III/IV II/III/IV	21	5	24	—	—	—	4	—	—	—	—	—	1	—	—	—
Miscellaneous (phage 187)	4	4		—	—	—	—	—	—	1	—	—	—	2	—	1
Miscellaneous (phage 81)	3	1		—	—	1	—	—	—	—	—	—	—	—	—	—
Non-typable	80	16	20	1	5	5	1	1	—	2	—	—	1	—	—	—
Total no. of strains	452	111	25	14	14	18	34	2	1	6	6	1	11	2	1	1

parison the results of enterotoxin tests on strains implicated in outbreaks of food poisoning are also given (Gilbert & Wieneke, 1973, and unpublished results).

Twenty-six of 112 strains isolated from raw foods showed enterotoxin production and of these 23 produced enterotoxin D alone or together with C. Of 183 strains from cooked foods, 63 produced one or more enterotoxins. Three enterotoxigenic strains were detected among 16 isolated from salami and pâté. Twenty-eight strains were isolated from cream and pastries filled with cream or artificial cream and nine of these produced enterotoxin. The three enterotoxigenic strains found among 50 isolates from raw milk all produced enterotoxin D. Enterotoxin was produced by seven of 63 strains from cheese and of these six produced D alone or together with B.

The admission swabs yielded 101 strains of which 38 produced one or more enterotoxins. Ninety-one enterotoxigenic strains were detected among 199 strains isolated from lesions.

One hundred and thirteen strains of 120 that were involved in 120 separate outbreaks of staphylococcal food poisoning were found to produce enterotoxin.

Only 11 of 240 enterotoxigenic strains isolated from foods or human sources produced enterotoxin E, alone or together with C or A and C.

The phage-typing patterns of the 452 strains isolated from foods were determined and Table 3 shows the enterotoxin production by staphylococci of different phage groups. Twenty-three–29 % of strains, which lysed with phages of group I or III or with phages of both these groups and 11 % of those in phage group II or IV produced enterotoxin. Four strains lysed by phage 187 (miscellaneous group) were all enterotoxigenic and three of these produced enterotoxin E together with other enterotoxins. Twenty per cent of the non-typable organisms produced enterotoxin. Thirty-nine strains of which 27 were isolated from milk or cheese were lysed by phage 42D (the only phage in group IV). Seven of these were enterotoxigenic and all produced D, alone or together with C.

DISCUSSION

The production of enterotoxins A, B, C or E was less frequent among strains isolated from raw foods, e.g. chicken, meat and milk, and from Cheddar cheese, than among those from cooked foods, e.g. chicken, meat and seafoods and from human beings. This difference between strains from raw foods, cooked foods and human beings is probably due to the fact that raw foods are likely to carry staphylococci mainly from the animal environment and cooked foods from the human environment; Casman *et al.* (1967) and Hájek & Maršálek (1973) found that production of enterotoxins A, B and C was less common among strains isolated from animals than among those from human beings.

Enterotoxin D production was more often found among strains isolated from raw meat and raw chicken than among those from cooked meat, cooked seafood and human beings. Staphylococci from cooked chicken resembled those from raw foods in this respect except that A or C were usually produced at the same time. It may be that enterotoxin D is more frequently produced by animal strains than by human strains.

Enterotoxigenic strains isolated from cheese and milk mainly produced enterotoxin D. Casman *et al.* (1967) also found that enterotoxin D production was more frequent among strains isolated from milk than production of A, B or C.

Enterotoxin A production occurred more often among the enterotoxigenic strains implicated in outbreaks of food poisoning, than among those isolated from foods or human beings unconnected with outbreaks. The frequency of enterotoxin B, C, D or E production was also different in staphylococci from outbreaks of food poisoning and in those from cooked foods and human beings. Thus, it appears that factors other than the presence of an enterotoxigenic staphylococcus in a food, play an important role in the development of enterotoxin in the food.

The results in Table 1 are similar to those of Casman *et al.* (1967), Untermann & Sinell (1970), Jarvis & Lawrence (1970), Terplan, Zaadhof & Bobeth (1971), Untermann (1972) and Hájek & Maršálek (1973).

Zak, Jeljaszewicz & Stochmal (1971) found that 75 % of strains isolated from faeces (mainly connected with diarrhoea) produced enterotoxin A, B or C or combinations of these. Terayama, Igarashi, Ushioda & Zen-Yoji (1972) detected enterotoxin production in 83 % of strains isolated from foods and 95 % of those from nasal and finger swabs and faeces from healthy human beings; the organisms were tested for the production of enterotoxins A–D only. Müller *et al.* (1973) reported that 74 % of strains isolated from nasal swabs of human beings and from clinical specimens produced enterotoxin when the organisms were tested for A, B and C and that 65 % of strains from the faeces of healthy human beings produced enterotoxin when tested for A, B, C, D or E; 25 % of the faecal strains produced enterotoxin E, alone or together with other enterotoxins.

The phage-typing pattern of a staphylococcus does not tell whether or not the organism produces an enterotoxin. Nevertheless in outbreaks of food poisoning phage-typing patterns are useful in the search for the implicated strains, which are in most cases lysed by phages of group III or I/III. Outbreaks are mainly caused by enterotoxins A, D, A + D or C + D and strains that were isolated from human beings and produced similar enterotoxins also yielded a high number (more than 75 %) that were lysed by phages of group III or I/III (M. T. Parker and J. H. Hewitt, to be published). The same results were obtained with strains isolated from routine samples of food, except for strains which produced enterotoxins C + D; only about half of these were lysed by phages of group III or I/III. The phage-typing pattern is also useful in the correlation of staphylococci isolated from foods, clinical specimens and food handlers in outbreaks of food poisoning.

Seven enterotoxigenic strains isolated from foods were lysed by phage 42D (the only phage in Group IV) or by this phage together with phages from other groups, and all produced enterotoxin D. One such strain isolated from a case of food poisoning also produced enterotoxin D. Of two similar strains isolated from lesions of hospital patients, however, one produced enterotoxin B and the other C. Toshach & Thorsteinson (1972) found one strain lysed by phage 42D (and also by phages of group III) that produced enterotoxin A.

Six strains* isolated from routine samples of food, three from foods implicated

* Two of these strains are not included in Table 3.

in outbreaks of food poisoning, three from hospital admission swabs, one from faeces and one from a lesion were lysed only by phage 187 (miscellaneous group). Thirteen of these produced enterotoxin (2 A, 5 (A+C), 1 C, 1 E, 3 (C+E) and 1 (A+C+E)). Toshach and Thorsteinson (1972) reported two strains implicated in two outbreaks of food poisoning that were lysed by phage 187 and both produced enterotoxin A+C. The number of strains tested is, however, too small to state whether there is a definite relationship between the lysis by phage 187 and the ability to produce enterotoxin.

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Mosquito-borne infections in Fiji

V. The 1971–73 dengue epidemic

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SUMMARY

A dengue epidemic due to type 2 virus involving some 3,400 cases began in Fiji early in 1971, had a peak during May, June and July, and cases have continued to occur with a low incidence during 1972 and 1973. Many of the notified cases showed classical dengue fever symptoms and there were no confirmed cases of haemorrhagic fever. A serological survey indicated that there had been at least 20,000 subclinical infections. It is probable that the virus was introduced to Fiji either through the port of Lautoka or Nadi international airport in February 1971. The normal travel patterns of residents must have spread the virus to all the more accessible localities but, with the exception of Rotuma, it caused infections only in areas where *Aedes aegypti* was available as a vector. There was no evidence that pre-existing dengue type 1 serum antibody gave any protection during this epidemic.

INTRODUCTION

There have been two major outbreaks of dengue in Fiji since the first epidemic was reported in 1885, one in 1930 and the other in 1943–44. Techniques for the isolation of dengue viruses were not available during these early epidemics, but a subsequent survey of sera for neutralizing antibody suggested that the 1943–44 epidemic was due to type 1 dengue virus (Miles *et al.* 1964). Notifications of dengue continued until 1966, but there is no serological evidence that dengue or any other arboviruses have been active in Fiji since 1950 (Maguire *et al.* 1971).

These authors also noted that the very high proportion of the population with no dengue antibody made Fiji a high risk area for a further dengue epidemic. Another dengue epidemic began early in 1971, reached a peak in the period May–July of that year, and continued through 1972 into 1973. Type 2 dengue virus was isolated from a number of cases. This report describes virological, serological, clinical and epidemiological aspects of the outbreaks.

MATERIALS AND METHODS

Notifications

Cases diagnosed on clinical grounds by medical officers were notified to the Fiji Medical Department. Data on age were available for more than half of the notifications. Blood samples were obtained from patients in the acute and convalescent phases of the illness from a number of cases in Suva, but very few from the other health districts.

Virus isolations

Material for virus isolation was injected intracerebrally (i.c.) into litters of mice less than 48 hours old. The mice were observed daily and any which became sick were killed and brain material was passaged again in mice. Brain smears from most mice which became sick were then tested for the presence of dengue virus antigen by staining with fluorescein-conjugated dengue virus antibody. For the later virus isolations it was found that more rapid and reliable results were obtained by examining stained brain smears from mice killed ten days after injection.

Haemagglutination inhibition (H.I.)

A dengue virus type 2 haemagglutinin for goose erythrocytes was prepared by sucrose-acetone extraction of infected suckling mouse brain (Clarke & Casals, 1958). Sera were treated with acetone to remove nonspecific inhibitors, and with goose red blood cells to remove nonspecific agglutinins. They were tested against 4–8 haemagglutinating doses of antigen in a microtitre system.

A recent survey showed that in 1969 less than 1 % of people born since 1948 had serum H.I. antibodies against dengue viruses (Maguire *et al.* 1971). It was therefore possible to investigate the origin and spread of this epidemic by testing sera taken randomly from people in this age group for the presence of dengue virus antibodies. An antibody incidence more than two standard deviations above 1 % indicated that dengue virus infections had occurred in the population from which the serum samples were drawn.

Experimental infection of mosquitoes

Laboratory-hatched mosquitoes were infected by intrathoracic injection or by allowing them to feed on viraemic mice, or on a mixture of mouse blood and infected mouse brain through a mouse tail-skin or from a cotton pledget. They were kept at 25° C–30° C for periods up to 23 days and were tested for the presence of virus either by staining salivary gland smears with fluorescein-conjugated dengue virus antibody, or by injecting mosquito extracts i.c. into suckling mice. Brain smears from any mice which became sick were stained with fluorescein-conjugated dengue virus antibody.

RESULTS

The first clinical case of dengue was diagnosed in March 1971 at Lautoka (Fig. 1) and was confirmed by demonstrating an increase in dengue virus antibody during the course of illness and convalescence. Six of 27 sera collected from young people

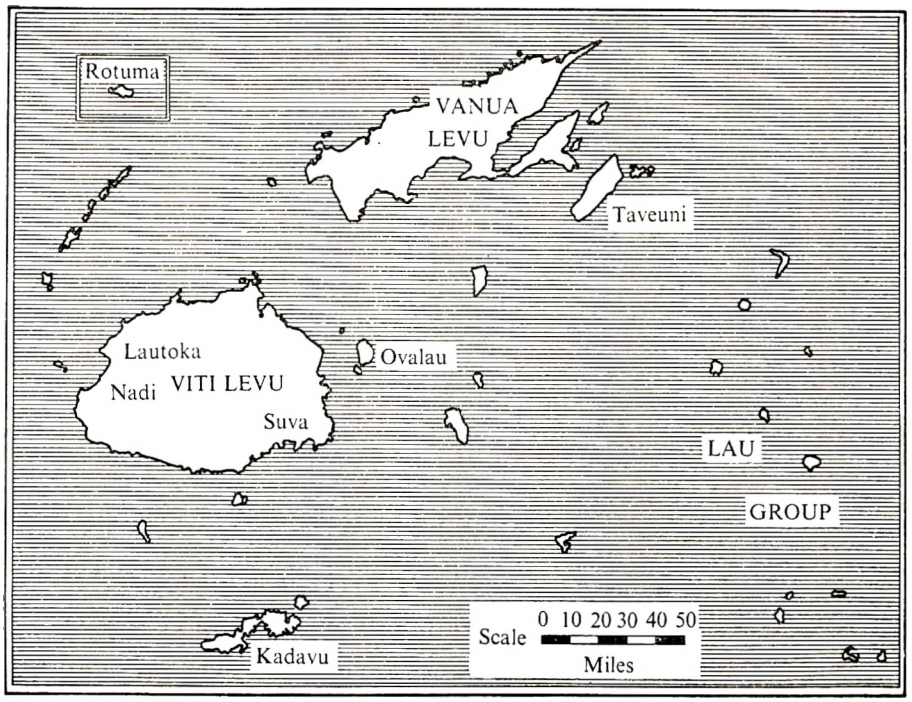

Fig. 1. Map of Fiji Islands. Rotuma is 240 miles NNW of Suva.

at Lautoka in February had HI antibodies to dengue virus, indicating that virus infections had occurred several weeks before the first diagnosed case.

By the end of March a number of other cases had been reported, but no formal notifications were received until May. The incidence of notifications and of laboratory-confirmation by virus isolation and serology is shown in Fig. 2. The number of cases notified remained high until the end of August when they began to decrease steadily to reach a minimum figure of 3 cases in January 1972. Dengue virus was isolated from the blood of one of these three cases. The number of notifications then increased each month to a second peak of 79 cases in April. Cases continued to be notified until April 1973 when the total had reached 3,413. The abrupt increase to 106 dengue notifications in the week ending 2nd September 1972 was probably due to erroneous diagnoses at the beginning of an influenza epidemic.

A substantial proportion of the cases showed the classical dengue fever syndrome but many cases were mild and difficult to differentiate clinically from cases of rubella which were occurring concurrently in 1971. Only three cases showing haemorrhagic manifestations were notified. All were adults. Attempts to isolate virus from the blood of these patients were unsuccessful, and no antibody was detected in their sera.

Seventeen virus isolations were made from blood or serum of acutely ill patients (Fig. 2). In ten cases the virus was shown to be dengue by examining infected mouse brain smears stained with fluorescein-conjugated antibody, and in 13 cases the patient developed dengue virus antibodies during the course of the illness. The H.I. antibody titres were much higher to type 2 than to type 1 virus. Dengue

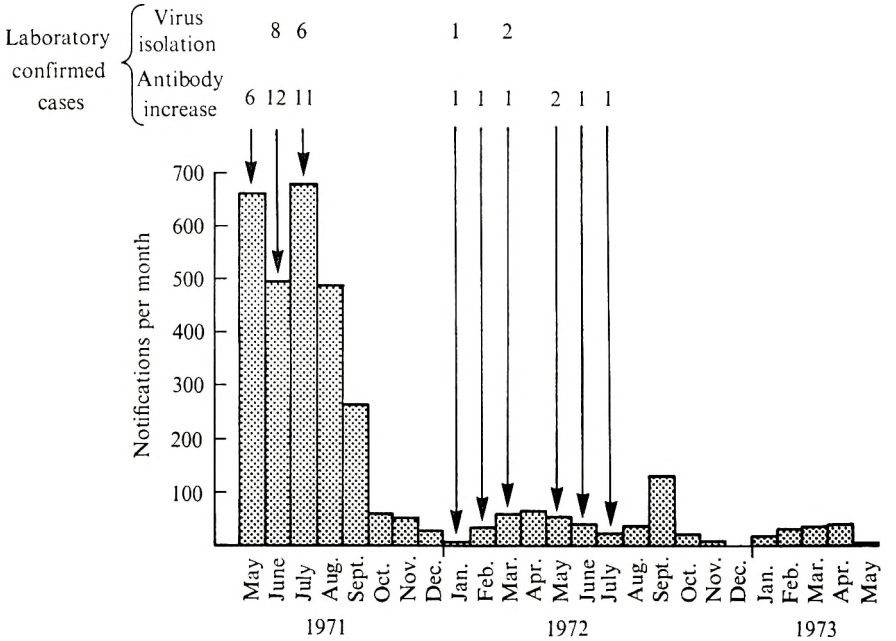

Fig. 2. Notification and laboratory-confirmed cases of dengue. May 1971 to May 1973.

virus infection was diagnosed in 36 other patients from whom no virus was isolated, on the basis of a four-fold or greater increase in H.I. antibody during the course of the illness. Serological studies confirmed by Dr R. L. Doherty of Brisbane and Dr Leon Rosen of Honolulu showed the virus strain to be type 2 dengue.

The virus was readily isolated in suckling mice as it caused illness about 14 days after primary i.c. inoculation. After ten passages in mouse brain the incubation time in mice was reduced to seven days. The virus grew in MK2 cells, the only cell type which has been tested.

Most of the notifications and clinical cases were from the Suva district, but the disease also affected people to varying degrees in other parts of Viti Levu, on the adjacent island of Ovalau, and on the remote island of Rotuma. The results of the survey of sera from people born since 1948 for the presence of dengue virus H.I. antibodies are shown in Table 1. In a number of areas the incidence of antibody positive sera is significantly higher than that prevailing in the age group before this epidemic. This confirms the evidence from notifications that dengue virus was active in all coastal areas in Viti Levu but not in the Western Highlands, and that infections also occurred on Ovalau and Rotuma but not on Vanua Levu or in the Lau Islands, and probably not on Kadavu. Twenty-six cases notified from Vanua Levu were shown serologically to be rubella virus infections.

The distribution of dengue notifications according to age, where information was available, is shown in Table 2. Seventy-three per cent of the notifications were from people over 20 years of age although they comprised less than 43% of the population, and 35·4% of them had dengue virus antibodies before the

Table 1. *Incidence of dengue virus H.I. antibody in sera taken at random from people born since* 1948

Area	Date collected	Number tested	Number positive	% positive	S.E.D. %*
All areas	1967–1969 (pre-epidemic)	4,886	29	0·6	—
Viti Levu					
Suva	Aug. 1971–1972	204	53	26·0	0·90
East Coast	1972	127	10	7·9	0·79
North Coast	1972–Jan. 1973	260	36	13·8	0·71
Lautoka	Feb. 1971	27	6	22·2	1·62
South West	1972	121	4	3·3	0·75
South Coast	1972	81	11	13·6	1·01
Western Highlands	1972	68	1	1·5	0·95
Vanua Levu	Jan.–June 1972	259	4	1·5	0·51
Ovalau	Aug. 1971	86	17	19·8	1·04
Lau group	1971–1972	455	2	0·4	0·37
Kadavu	June 1972	120	3	2·5	0·74
Rotuma	Sept. 1972	191	82	42·9	1·08

* Standard error of difference between % positive for pre-epidemic and post-epidemic populations.

Table 2. *Dengue notifications by age**

Age (years)	Percentage of population. (1966 census) [Total 476,727 persons]	Approximate % of age group with dengue virus antibodies before 1970	Number of notifications	Percentage of notifications
0–19	57·2	0·6	390	27
20–39	26·2	21·0 } 35·4	742	51
40+	16·6	54·3 }	320	22

* No age information was available from 1,961 notifications.

onset of the epidemic. Serological surveys of young people have indicated that in epidemic areas up to 43% of people born since 1948 were infected during the epidemic (Table 1).

Except on Rotuma, dengue occurred only in areas where *Aedes aegypti* was present. A $2\frac{1}{2}$ day search of the most likely *Ae. aegypti* breeding places on Rotuma revealed *Ae. rotumae* as the only *Stegomyia*. Since there is evidence that *Ae. polynesiensis* and *Ae. pseudoscutellaris* had been dengue virus vectors in an earlier epidemic (Maguire *et al.* 1971) experiments were performed to compare the infectivity of an epidemic strain of virus for *Ae. aegypti* and *Ae. pseudoscutellaris*. The results are shown in Table 3. It was found that 40% or more *Ae. aegypti* could be infected by feeding them on blood containing $10^{6\cdot5}$LD50 of dengue virus per ml. but not one of 59 *Ae. pseudoscutellaris* became infected when fed in the same way on a virus preparation containing $10^{6\cdot0}$ LD50 per ml. Further experiments with *Ae. aegypti* showed that they could be infected by feeding on viraemic mice in which the amount of dengue virus was less than $10^{0\cdot5}$LD50 per ml. of blood,

Table 3. *Mosquito infection experiments with Fiji dengue virus strain* 1070

Mosquito species	Method of infection	Virus content of material used (log_{10}LD 50/ml.)	Days after infection	No. infected/ No. tested	Method used to detect virus*
Ae. pseudo-scutellaris	Fed through mouse tail skin	6·0	17	0/32	s.m.i.
Ae. pseudo-scutellaris	Fed on cotton pledget	6·0	20	0/27	s.m.i.
Ae. aegypti	Fed through mouse tail skin	6·5	18	10/23	s.m.i.
Ae. aegypti	Fed on cotton pledget	6·5	18	6/15	s.m.i.
Ae. aegypti	Fed on viraemic mice	< 0·5	23	2/15	s.m.i.
Ae. aegypti	Intrathoracic injection	5.6	20	3/3	f.a.

* s.m.i. = suckling mouse inoculation.
f.a. = fluorescent antibody staining of salivary glands.

and that 20 days after intrathoracic injection dengue virus was concentrated in the salivary glands. Laboratory colonies of *Ae. polynesiensis* and *Ae. rotumae* were not available for infectivity studies.

DISCUSSION

Serological evidence indicates that in Fiji the only arboviruses which have commonly infected man are dengue viruses. The prevalence of antibody in relation to year of birth indicates that dengue virus epidemics have occurred from time to time and that little or no infection has occurred between these epidemics. The last dengue outbreak was in 1943–44 and serological responses of individuals involved in that outbreak strongly suggest that it was due to type 1 dengue virus (Miles *et al.* 1964; Maguire *et al.* 1971).

Dengue recurred in 1971 and, although the disease was first diagnosed in Lautoka in March, serological studies indicated that it had been present for some weeks at that time. The epidemic rapidly built up and reached a peak in June and July. The limited number of notifications in 1972 and 1973 brings the total number notified to 3,413 (Fig. 1).

Although there were many classical dengue cases, there were also very many mild atypical cases, and serological studies show that there were large numbers of subclinical immunizing infections (Table 1). When there was a concurrent rubella epidemic the clinical differentiation of mild dengue from rubella proved difficult, and the beginning of an influenza epidemic led to a sudden rise in dengue notifications. The difficulty of making a differential diagnosis between dengue and other acute febrile infections is well recognized (Clarke & Casals, 1965), and our problems merely emphasize this fact.

Three cases of possible haemorrhagic dengue were reported but it could not be shown that any of them were due to infection with dengue virus. This is in contrast to the 1971 dengue 2 virus epidemic in Tahiti in which a number of cases with haemorrhagic manifestations were observed (Moreau, Rosen, Saugrain & Lagraulet, 1973). However, Tahiti has had three dengue outbreaks since 1960, and Moreau *et al.* (1973) noted that their observations were consistent with the hypothesis that haemorrhagic manifestations due to infection with dengue viruses are the results of a secondary response in a previously sensitized individual. Nevertheless other factors must be involved as no haemorrhagic manifestations were observed by Likosky *et al.* (1973) in a similar situation in Puerto Rico where a dengue type 2 epidemic in 1968–69 followed a dengue type 3 epidemic in 1963.

The age distribution of notified cases was surprising in view of the incidence of dengue virus antibody before the epidemic. Although approximately 57 % of the population had been born since the 1943 epidemic, and less than 1 % of them had dengue virus antibodies, only 27 % of the notifications came from this group. On the other hand more than half the notified cases were in the 20–39 year age group which formed about 26 % of the population, and there was serological evidence that 21 % had been exposed to dengue viruses before this outbreak. Although the infection rate was higher than indicated by the notifications, due to subclinical infections, the age distribution of notified cases suggests that pre-existing dengue virus antibodies in people born before 1950 gave little protection against infection in this epidemic.

The virus was probably introduced into the country in February either through the port of Lautoka or through the international airport at Nadi, which is only about 20 miles from Lautoka. The origin of the virus is unknown, but during 1971 and 1972 outbreaks of dengue type 2 occurred in Tahiti (Moreau *et al.* 1973), Gilbert and Ellice Islands, New Hebrides, Western Samoa, New Caledonia, Niue and New Britain (Marshall & Hawkes, 1972). After the first case was diagnosed at Lautoka in March the epidemic spread during the next four months to most coastal areas of Viti Levu, to the adjacent island of Ovalau and to Rotuma, 240 miles to the north. Although the normal travel patterns of residents must have introduced dengue virus into all the more accessible localities in Fiji so that all human-biting mosquitoes would have had adequate opportunities of becoming infected in the field, the geographical distribution of the notifications indicates that, except for Rotuma, the disease spread only to areas where *Ae. aegypti* occurs.

Mosquito infectivity experiments showed that the strain of dengue virus responsible for this epidemic could infect *Ae. aegypti* but not *Ae. pseudoscutellaris*. Technical problems prevented testing the infectivity of the virus for the other possible vector mosquito, *Ae. polynesiensis*.

In apparently having only one virus vector, this dengue epidemic differs from previous outbreaks. The distribution of dengue virus antibodies amongst the population in 1969, particularly in Vanua Levu and Taveuni, indicates that in previous outbreaks dengue virus must have been readily transmitted not only by *Ae. aegypti* but also by *Ae. polynesiensis* and *Ae. pseudoscutellaris* (Maguire *et al.* 1971).

In view of the apparent ineffectiveness of type 1 serum antibodies in protecting people against infection with this virus, it is fortunate that the strain was a relatively avirulent one. It caused little serious illness and the subclinical infections stimulated the production of serum antibodies in many people born since the last epidemic. The increasing efficiency of communication between Fiji and areas where dengue viruses are endemic make it unlikely that another 28 years will elapse before the next introduction of a dengue virus into Fiji. As this virus may be more virulent, close surveillance for future outbreaks should be maintained.

We are grateful for facilities provided by the Medical Department of Fiji, and for assistance from Mr M. G. R. Ross and the staff of the Wellcome Virus Laboratory, Suva, Fiji.

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The bacteriological status of a specific-pathogen-free animal production building and of its staff and the microbiological integrity of the animals one year after the building was commissioned

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SUMMARY

Bacteriological contamination of a specific-pathogen-free (SPF) animal production building was more extensive after one year of operation than at the time of commissioning. Throat and nose swabs taken from the staff yielded a number of undesirable organisms, in particular, on one occasion, a Group A streptococcus. It is recommended that a closer bacteriological scrutiny of the staff of SPF production buildings ought perhaps to be made in the light of these findings.

INTRODUCTION

The bacteriological status of a new SPF animal production building was assessed before and after fumigation (Taylor, 1974). During the subsequent twelve months large numbers of animals have been produced, some 4550 entries through the barrier have been made and various quantities of maintenance materials have been introduced.

This paper records the results of an assessment after twelve months of the microbiological status of the animals and of the bacteriological status of the building and staff.

MATERIALS AND METHODS

Ninety-five sites in the building were examined by the method described by Taylor (1974). The cultures obtained after 72 hr. incubation at 37° C. were subcultured to 5 % horse-blood agar plates and incubated for a further 36 hr. The organisms thereby isolated were identified according to their morphology and reaction to routine laboratory tests.

Animals

The animals in the building were examined each month for the presence of proscribed microflora for animals of **** rating, according to the methods recommended by the MRC Laboratory Animals Centre, Accreditation Microbiological Advisory Committee (MRC/LAC, AMAC). Briefly, this publication recommends methods of isolation and examination for proscribed microflora and indicates the preferred sites of culture. Pathogenic or potentially pathogenic organisms are catalogued in another MRC publication (MRC, 1972).

Table 1. *Results of bacteriological examination of various sites in a S.P.F. animal production building one year after it had been commissioned*

	Sites in each area		Aerobic spore-bearers	*Ps. aeruginosa*	*Proteus* spp.	*E. coli*	*Aerobacter* spp.	Strep. viridans	Strep. group D	Coagulase neg. staph.	Diphtheroids
	No.	Pos.									
Animal rooms (10)	50	42	8	4	1	5	3	5	1	13	1
Service area	10	6	2	2	—	1	1	1	1	—	—
Office	5	3	—	—	—	1	—	1	—	1	—
Mess	5	5	1	3	—	—	1	—	—	3	1
Store	5	2	—	1	—	—	—	—	—	2	—
Toilets											
Men's (inside)	5	4	—	—	—	—	—	1	—	4	—
Men's (outside)	5	4	2	1	—	—	—	—	—	1	—
Ladies' (inside)	5	3	—	—	—	—	—	—	—	3	—
Ladies' (outside)	5	5	2	1	—	1	1	1	—	1	—
Totals	95	74	15	12	1	8	6	9	2	28	2

Table 2. *Results of the microbiological and parasitological examination of random samples of animals produced in a S.P.F. animal production building up to one year after it was commissioned*

	Mice	Rats	Guinea-pigs	Total
Staphylococci				
Coagulase-positive	1 (2)	0	0	1 (1)
Coagulase-negative	5 (10)	1 (5)	1 (13)	7 (9)
Streptococci				
Viridans	37 (77)	16 (80)	8 (100)	61 (80)
Group C	0	1 (5)	0	1 (1)
Group D	0	1 (5)	0	1 (1)
Haemophilus influenzae	0	4 (20)	0	4 (5)
Proteus spp.	23 (48)	17 (85)	1 (13)	41 (54)
Escherichia coli	23 (48)	16 (80)	8 (100)	47 (62)
Diphtheroid bacilli	3 (6)	0	2 (25)	5 (7)
Lactobacilli	44 (92)	8 (40)	2 (25)	54 (71)
Mycoplasmas	0	0	0	0
Ecto-/endo-parasites	0	0	0	0
Significant serum titres against				
Sendai virus	0	0	0	0
Leptospiras*	0	0	0	0
Total animals tested	48	20	8	76

Figures in parentheses are percentages.
* Serum samples tested against 16 different antigens.

Staff

At the time the animals were monitored, throat and nose swabs from members of the staff servicing the unit were spread on 5 % horse-blood agar plates and incubated for up to 36 hr. at 37° C. Organisms recovered were identified according to accepted bacteriological principles.

RESULTS

The building

Organisms were isolated from 74/95 (78 %) of sites examined (Table 1). This compares with 13/100 (13 %) of sites examined when the building was brought into commission after fumigation (Taylor, 1974). The types of organisms recovered were similar to those present at the time of the first examination. Comparison of these two examinations shows that there was a 13 % increase in the number of sites contaminated with Gram-negative flora (3/13 23 % 1973:27/74 36 % 1974) and an 11 % decrease in the number of sites contaminated with Gram-positive cocci (staphylococci and streptococci) (8/13 64 %:39/74 53 %) during the year. The percentage of aerobic spore-bearing organisms remained constant and only a few diphtheroids were identified.

The animals

The results of tests on 76 animals are given in Table 2. The background flora was found to be fairly constant in all species, the only variation being in the rats from which groups C and D streptococci and *Haemophilus* species were isolated.

Table 3. *Results of the bacteriological examination of nose and throat swabs from staff servicing the S.P.F. animal production building*

Organisms isolated	A*		B		C		D		E		F		G		H		I		Totals	
	N	T†	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T
Staphylococci																				
Coagulase positive	2	—	1	—	5	2	—	—	1	—	2	1	—	1	—	—	—	—	11	4
Coagulase negative	4	—	3	1	2	1	2	—	3	—	1	—	1	—	—	—	1	—	17	2
Streptococci																				
Viridans	2	6	—	7	1	10	1	5	—	4	1	1	2	2	1	1	—	1	8	37
Group A‡	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	1
Group C	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Not groupable	—	3	—	1	—	1	—	—	—	1	—	—	—	—	—	—	—	—	—	6
Neisseria catarrhalis	—	—	—	2	—	1	—	1	—	1	—	—	—	—	—	—	—	—	—	5
Haemophilus influenzae	1	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—
Lacobacilli	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Proteus	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—
E. coli	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	1	—
Diphtheroids	1	1	1	2	—	1	—	—	—	1	1	—	—	—	—	—	—	—	3	5
Staphylococcus albus	—	—	2	3	2	3	2	1	—	—	—	—	1	2	1	—	—	—	8	9

* Staff reference: A–F = permanent staff; G–I = occasional staff. At no one time during the period of the observations were all nine members of the staff available for swabbing at the same time, owing to absence through illness or other cause.

† N, Nose; T, throat.

‡ Proscribed organism (grouped by Fuller's method).

The group C streptococci and the *Haemophilus* may have been of human origin (see Table 3). There was no evidence of ecto- or endo-parasites nor of *Mycoplasma* species, and significant antibody titres to Sendai virus or leptospira were not demonstrated. Screening of mice for persistent tolerant infection (PTI) to lymphocytic choriomeningitis (LCM) virus in mice showed that all of 38 mice tested were susceptible to challenge with the Pirbright LCM strain and no immunity was demonstrated. The colony from which these mice were derived may therefore be considered to be LCM-free.

The staff

Eleven of 46 nose swabs and 4/46 throat swabs of staff examined on ten occasions yielded coagulase positive staphylococci (Table 3); *Haemophilus influenzae* was isolated from the noses of two persons; Group A streptococci (proscribed for **** animals) were isolated from the throat of another member of staff, as also was a Group C streptococcus; *Neisseria catarrhalis* was isolated from throat swabs on five occasions. With the exception of the Group C streptococcus and haemophilus, none of the fore-mentioned organisms was recovered from the animals.

DISCUSSION

During the first year of operation of the SPF barrier system for producing mice, rats and guinea-pigs at this Institute, no proscribed organisms were isolated from the animals or from various sites within the building. However, a member of the staff contracted a throat infection associated with Group A streptococci; spread of this organism to the animals was avoided by the person reporting the condition immediately and remaining outside the barrier until it had abated.

A change in the bacteriological status of the building compared with that of the year previously, was of a minor nature and consisted of an alteration in the degree of contamination rather than the occurrence of new species. This was evidenced by the increase of the Gram-negative coliform types and the decrease in the Gram-positive cocci. It is possible that this situation may be attributed to two causes: (1) the increase in the number of animals, and therefore of the faecal contamination of the environment that their routine husbandry creates and (2) the regular and systematic use of a surface acting ampholytic biocide as a cleaning/disinfecting agent. Unpublished work in the Disinfection Reference Laboratory, Colindale, has shown it to be comparatively ineffective against Gram-negative flora although more active against Gram-positive organisms. These agents are more seriously inactivated by hard water and by organic material than are most other disinfectants. They are also severely inactivated by wood, cork, rubber, cotton, nylon and certain plastics (J. C. Kelsey & I. M. Maurer, personal communication). Those responsible for bacteriological tests of surfaces which have been treated with ampholytic disinfectants may be reminded that these agents are highly bacteriostatic. They must be neutralized to enable surviving organisms to grow and make their presence known (MacKinnon, 1974).

The appearance in the rats of *Haemophilus* species and Group C streptococci is

of particular interest because, although not proscribed organisms for **** animals, they have not hitherto been observed in our SPF animals. Both of these organisms were isolated from two members of staff about two months before their appearance in the rats, suggesting infection from a human source. If this were the case it demonstrates the ease with which cross-infection occurs and emphasizes the extreme care which is required in monitoring staff for proscribed organisms and in selecting staff of high integrity, who can be relied upon to bring apparently minor cases of illness to the attention of their supervisor, before entry to the unit is contemplated. *Neisseria catarrhalis* was not observed in any of the animals although it was isolated from the staff on five occasions.

The recovery of proscribed micro-organisms from a member of the staff indicates the need for frequent bacteriological examination of nose and throat swabs (weekly, perhaps, instead of monthly). There would also appear to be a strong case for conducting a microbiological survey of the building such as that described in this paper, at least annually. The work involved is little compared with the tremendous cost if a 'breakdown' should occur.

I wish to record my gratitude to Mr H. H. Skinner, F.R.C.V.S., of the Animal Virus Research Institute, Pirbright, for testing the mice for LCM susceptibility; to the staff of the S.P.F. Building for their willing co-operation in this work, and to Mr D. Hendry for his helpful technical assistance.

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Further studies of *Escherichia coli* in babies after normal delivery

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SUMMARY

Previous work showed that on the basis of O serotyping alone of *Escherichia coli*, the majority of babies acquired the same O serotype as was found in the stools of their respective mothers. Further characterization of the *E. coli* by H serotyping, determination of their antibiotic resistance and ability to ferment six carbohydrates showed that in the majority of cases the previous results were confirmed. In a minority of cases this further testing showed that the strains were not identical. In some instances a number of strains isolated from the same pair showed different combinations of the markers used.

INTRODUCTION

Previous work (Bettelheim *et al.* 1974) showed that from the faecal flora from 22 out of 28 babies *Escherichia coli* carrying the same O antigen as that found amongst the *E. coli* of the maternal faeces were isolated. This work describes the further characterization of these 2525 strains. Strains were typed according to their H antigen, their ability to ferment six carbohydrate substrates and their antibiotic resistance. The maternal and baby strains were then again compared.

MATERIALS AND METHODS

The collection of strains and O serotyping has been described previously (Bettelheim *et al.* 1974). The numbers used to identify the mothers and their babies are the same as those used previously.

H antigen determination

The H antigens of all strains were determined after achieving full motility (Chandler & Bettelheim, 1974). Strains consistently unable to develop motility as judged by microscopical observation were considered non-motile.

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Table 1. *Isolation of identical types of* Escherichia coli *from mothers and their babies*

Patient no.	Common strain in maternal and neonatal stool			
	O antigen	H antigen	Antibiotic resistance	Carbohydrates fermented
2	3	2	Su	Du, Ma
	81	27	F/S	Ma, Rh
21	18	14	F/S	Ma, Rf, Rh, Sc
	58	40	F/S	Ma, Rf, Rh
	R	14	F/S	Ma, Rf, Rh, Sc
35	11	4	S, Su	Du, Ma, Rh
	R	4	S, Su	Du, Ma, Rh
37	25	1	F/S	Ma, Rh
	NT	8	F/S	Du, Ma, Rf, Rh, Sc
1	1	NM	F/S	Du, Ma, Rh, Ss
26	1	NM	F/S	Ma, Ss

Carbohydrate fermentation

The ability of the strains to ferment dulcitol (Du), maltose (Ma), raffinose (Rf), rhamnose (Rh), sorbose (Ss) and sucrose (Sc) were examined (hereafter the abbreviations in brackets are used to label these markers). Bettelheim & Taylor (1969) found that of 16 carbohydrates used, the above six gave most differentiation between strains. Strains were inoculated by a multiple point inoculator onto agar plates of Oxoid Blood Agar Base (CM 55) containing 1 % of the substrate (w/v) and neutral red (6×10^{-5} %, w/v, final concentration). Plates were incubated at 37° C. and examined after 24, 48 and 72 hr.

Antibiotic resistance

Approximately ten organisms of each strain were inoculated by a multiple point inoculator on agar plates (Oxoid DST agar, CM 261) containing 4 % lysed blood and one of the following drugs: ampicillin (A), 25 μg./ml., streptomycin (S) 50 μg./ml., tetracycline (T) 10 μg./ml., chloramphenicol (C) 25 μg./ml., kanamycin (K) 10 μg./ml., sulphadimidine (Su) 100 μg./ml., nalidixic acid (Nal) 25 μg./ml., trimethoprim (Tr) 2 μg./ml. and gentamicin (G) 8 μg./ml. The characters shown in brackets are used as abbreviations throughout. Strains which are sensitive to all drugs tested are designated fully sensitive (F/S).

RESULTS

The further testing of the strains from six mothers and their babies showed no differences with respect to any of the characters tested (Table 1).

Strains from six further mothers and babies still showed that the majority of babies' stool strains were identical with the maternal ones. However, there were a minority of strains from the babies which differed from the maternal ones by one or more markers (Table 2).

Table 2. *Patients in whom the majority of strains of* Escherichia coli *isolated from mothers and their babies were identical but related strains were also isolated in small numbers*

Patient no.	Common O antigen	Strains isolated	Strains isolated from: Mother stool	Baby mucus	Baby stool
5	42	O42:H−:F/S:Du, Ma, Rf, Rh, Ss, Sc	1	0	1
		O42:H8:F/S:Du, Ma, Rf, Rh, Ss	0	2	0
	78	O78:H−:F/S:Du, Ma, Rf, Rh, Ss, Sc	30	11	48
		O78:H8:F/S:Du, Ma, Rf, Rh, Ss	0	1	0
		O78:H18:S, Su:Du, Ma, Rf, Rh, Ss, Sc	0	2	0
		O78:H18:F/S:Du, Ma, Rf, Rh, Ss, Sc	1	0	0
17	71	O71:H11:F/S:Ma, Rh	7	0	42
	81	O81:H−:F/S:Ma, Sc	3	0	1
		O81:H−:F/S:Ma, Rh, Ss	2	5	0
		O81:H11:F/S:Ma, Rh	0	0	1
18	48	O48:H32:F/S:Du, Ma, Rf, Rh, Ss	2	8	24
		O48:H32:F/S:Ma	0	0	1
33	84	O84:H2:Su:Du, Ma, Rf, Rh	5	0	5
		O84:H2:Su:Du, Ma, Rf, Sc	0	0	2
13	7	O7:H30:F/S:Ma	1	NS*	0
		O7:H−:F/S:Ma, Rh	1	—	0
		O7:H−:F/S:Ma, Rf, Rh, Ss, Sc	6	—	44
	82	O82:H31:A:Du, Ma, Rf, Rh	1	—	0
		O82:H31:F/S:Du, Ma, Rf, Rh	0	—	6
22	6	O6:H1:F/S:Du, Ma, Rh, Ss	14	0	30
		O6:H−:A:Du, Ma, Rf, Rh	1	0	0

* NS = no specimen obtained.

Table 3. *Mothers and their babies colonized with identical O serotypes of* Escherichia coli. *Further typing showed the majority of strains to be related but not identical*

Patient no.	Common O antigen	Strains isolated	No. of strains isolated from: Mother stool	Baby stool
6	38	O38:H25:F/S:Ma, Rf, Rh, Ss, Sc	1	0
		O38:H37:F/S:Du, Ma, Rh, Ss	9	0
		O38:H−:F/S:Du, Ma, Rh, Ss	1	4
		O38:H4:F/S:Du, Ma, Rh, Ss	0	56
7	42	O42:H7:F/S:Du, Ma, Rf, Rh, Ss	1	0
		O42:H19:F/S:Du, Ma, Rf, Rh, Sc	1	68
		O42:H19:A:Du, Ma, Rf, Rh, Sc	7	0
		O42:H19:F/S:Du, Ma, Rh, Sc	12	0
		O42:H19:A:Du, Ma, Rh, Sc	1	0
	R	R:H7:F/S:Du, Ma, Rf, Rh, Ss	6	0
		R:H19:F/S:Du, Ma, Rf, Rh, Sc	0	1

No strains of *E. coli* were isolated from the mucus of these babies.

Table 4. *Identification of rough and non-typable* Escherichia coli *from mothers and their babies*

Patient no.	Common O antigen	Strain isolated	No. of strains isolated from: Mother stool	Baby mucus	Baby stool
15	R	R:H−:F/S:Ma, Rh, Ss	5	0	0
		R:H−:S:Ma, Rh, Ss	2	0	0
		R:H−:Su:Ma, Rh, Ss	7	0	0
		R:H7:F/S:Du, Ma, Rh, Ss	0	0	50
19	R	R:H7:F/S:Du, Ma, Rf, Rh, Ss	8	0	50
		R:H7:T:Du, Ma, Rf, Rh, Ss	1	0	0
10	NT	OX*:H10:F/S:Ma	1	NS†	3
		OX:H10:T:Ma	37	—	37
29	42	O42:H2:F/S:(strain died)	1	0	0
		O42:H37:F/S:Ma, Rh, Sc	0	0	1
	79	O79:H7:F/S:Ma, Rf, Rh, Sc	1	0	0
		O79:H7:F/S:Du, Ma, Rh, Sc	0	0	1
	R	R:H37:T:Ma	1	0	0
		R:H37:T:Ma, Rh, Sc	1	0	0
		R:H37:F/S:Ma, Rh, Sc	1	0	0
		R:H7:F/S:Ma, Rh, Sc	1	0	0
		R:H52:F/S:Du, Ma, Rf, Rh	2	0	0
		R:H7:F/S:Du, Ma, Rf, Rh	0	1	0
		R:H37:F/S:Du, Ma, Rf, Rh	0	1	0
		R:H7:F/S:Du, Ma, Rh, Sc	0	1	1
		R:H−:F/S:Ma, Rh, Ss	0	12	21
	NT	OY:H2:F/S:Ma, Rh, Sc	5	0	0
		OY:H2:F/S:Du, Ma, Rh, Sc	9	0	32
		OY:H2:F/S:Du, Ma, Rf, Rh	2	1	0
		OY:H−:F/S:Du, Ma, Rf, Rh	3	0	0
		OY:H−:F/S:Du, Ma, Rh, Sc	0	0	3
		NT:H2:F/S:Du, Ma, Rh, Sc	0	0	1
23	R	R:H7:F/S:Du, Ma, Rh	7	4	0
		R:H7:F/S:Du, Ma, Rf, Rh, Sc	0	1	0
		R:H52:F/S:Ma	1	0	0
		R:H5:F/S:Du, Ma	1	0	0
		R:H−:F/S:Du, Ma, Rf, Rh, Sc	4	0	1
		R:H−:F/S:Ma, Sc	1	2	0
	NT	NT:H4:F/S:Ma, Rh, Ss, Sc	2	0	0
		NT:H−:F/S:Du, Ma, Rf, Rh, Sc	1	0	0
		NT:H−:F/S:Du, Ma, Rf, Rh, Ss, Sc	1	0	0
		NT:H−:F/S:Du, Ma, Rf, Sc	1	0	0
		NT:H−:F/S:Du, Ma, Rf, Rh	1	0	0
		NT:H−:F/S:Ma	1	0	0
		OX:H10:F/S:Ma	0	0	1
		OX:H10:T:Ma	0	0	18
27	27	O27:H−:F/S:Ma, Rh	1	2	35
	108	O108:H19:F/S:Du, Ma, Sc	2	0	0
		O108:H−:F/S:Du, Ma, Sc	0	1	1
		O108:H−:F/S:Du, Ma, Rf, Sc	0	0	1
	R	R:H4:F/S:Du, Ma, Sc	1	0	0
		R:H−:F/S:Ma, Rf, Rh, Sc	1	0	0
		R:H−:F/S:Ma	0	1	6

Table 4 (*cont.*)

Patient no.	Common O. antigen	Strain isolated	No. of strains isolated from Mother stool	Baby mucus	Baby stool
27	NT	NT:H34:F/S:Ma, Sc	3	1	1
		NT:H4:F/S:Du, Ma, Sc	2	3	0
		NT:H−:F/S:Du, Ma, Sc	1	0	0
		NT:H4:F/S:Ma, Ss, Sc	1	1	6
		NT:H11:F/S:Ma, Sc	0	1	1
		NT:H8:F/S:Ma, Rh	0	1	0
		NT:H9:F/S:Ma, Ss, Sc	0	0	1
		NT:H−:F/S:Ma	0	0	2
30	141	O141:H2:F/S:Du, Ma, Rh, Sc	8	0	27
	NT	NT:H30:F/S:Du, Ma, Rf, Rh, Sc	1	0	0
		NT:H12:F/S:Ma, Rf, Rh, Sc	1	0	0
		NT:H2:F/S:Du, Ma, Rh, Sc	0	0	3

* OX = O162 – see Discussion.
† NS = no specimen obtained.

From another two mothers and babies identical strains were found but only in minority numbers. The majority of strains isolated varied from one another by one or more markers, although the O type was the same (Table 3).

In one case (patient 31) there were three strains all with the O9 antigen isolated from the mother: (i) O9:H14:F/S:Du, Ma, Rf, Rh; (ii) O9:H−:F/S:Du, Ma, Rf, Rh; and (iii) O9:H−:F/S:Du, Ma, Rh – no strains of *E. coli* were isolated from the baby's mucus and the only strain isolated from the baby's faeces, although serotype O9 was O9:H10:F/S:Du, Ma, Rf, Rh. Many strains from both mother and baby were tested against both H antisera, which are not related (Edwards & Ewing, 1972) and no cross-reaction was found.

In the previous study (Bettelheim *et al.* 1974) rough or non-typable strains were considered similar if they were found in specimens from one mother and her baby. There were two patients (15 and 19) where rough strains were the only strains common to mother and baby. Further studies showed that in patient 19 the strains were in fact similar but in patient 15 the strains were quite different (Table 4). In two patients (10 and 29), whose predominant strains were non-typable by O serotyping, it was noted that the strains carried the same H antigen. O antiserum was, therefore, raised to an isolate from each of these patients and all other non-typable strains tested against them. This led to the identification of two more O serotypes called X and Y for the purpose of this paper. In the other patients (23, 27, 30) with non-typable strains, the other markers were too dissimilar (Table 4) to warrant preparing further sera.

DISCUSSION

O serotyping has been extensively used in the study of the spread of *Escherichia coli* in man (Linzenmeier, Freislederer, Apak & Metz, 1961; Linzenmeier, 1962; Ewing, 1962; Turck, Petersdorf & Fournier, 1962; Nejedla, Srajbr & Lanc, 1967;

Table 5. *Examples in which further typing indicated that strains thought to be different merely showed O–R variation*

Patient no.	Strains isolated	No. of strains isolated from: Mother stool	No. of strains isolated from: Baby stool
18	O48:H32:F/S:Du, Ma, Rf, Rh, Ss	2	24
	R:H32:F/S:Du, Ma, Rf, Rh, Ss	0	32
	O48:H32:F/S:Ma	0	1
	R:H32:F/S:Ma	1	1
35	O11:H4:S, Su:Du, Ma, Rh	16	54
	R:H4:S, Su:Du, Ma, Rh	13	6

Gruneberg, Leigh & Brumfitt, 1968; Cooke, Ewins & Shooter, 1969), animals (Sojka & Carnagham, 1961; Glantz, Narotsky & Bubash, 1962; Soderlind, 1965; Gossling & Rhoades, 1966; Hemsley, Barnum & Ingram, 1967; Shooter, Cooke, Rousseau & Breaden, 1970) and the environment (Muller, 1967; Cooke *et al.* 1970). At first only a limited number of O antisera were available but nevertheless this initiated these studies. As more types were identified, the studies could be extended. Recently, with 152 O antisera, it seemed that extensive epidemiological investigation might be more successful. This study shows that although O serotyping alone gave meaningful answers in a majority of cases and no further information was obtained from examining strains in more detail, there was a significant minority of cases in which a common O serotype was found but this did not indicate spread of the same strain.

The use of further O antisera prepared against untypable wild strains led to further differentiation of strains. Both X and Y antisera were tested against all known O antigens and no significant cross-reactions were detected. Strain X, representatives of which were found in a number of patients, has been forwarded to Dr F. Ørskov (International Escherichia Centre, Copenhagen) who has confirmed that it is a new serological type and will be designated O162. Strain Y, which was only found in one patient, has not been sent to Dr F. Ørskov, as current policy of the International Centre is not to put up new antigen numbers unless these are of special importance in clinical medicine or science (Ørskov, Ørskov & Rowe, 1973).

Furthermore, the use of both H and biotyping of some rough strains showed them to have the same markers as O types isolated from the same mother or baby. This O–R variation is well established and therefore the further typing enabled the detection of further common strains. Examples of this (patients 35 and 18) are shown in Table 5.

In other cases a number of strains isolated from the same pair showed different combinations of markers (Table 6). Furthermore, the same phenomenon was seen in strains isolated in a 10-day period from several patients (Table 7). In the latter case the markers H38 and H52 in our experience are most unusual (Bettelheim, 1969, Ph.D. thesis; Bettelheim, in preparation). On the other hand, there have been a number of instances of colonization of different mothers and babies with

Table 6. *Examples of strains showing some markers in common from mother and baby pairs*

Patient no.	Strains isolated	No. of strains isolated from Mother stool	Baby mucus	Baby stool
5	O78:H−:F/S:Du, Ma, Rf, Rh, Ss, Sc	30	11	48
	O78:H18:F/S:Du, Ma, Rf, Rh, Ss, Sc	1	0	0
	O78:H18:S, Su:Du, Ma, Rf, Rh, Ss, Sc	0	2	0
	O78:H8:F/S:Du, Ma, Rf, Rh, Ss	0	1	0
	O42:H−:F/S:Du, Ma, Rf, Rh, Ss, Sc	1	0	1
	O42:H8:F/S:Du, Ma, Rf, Rh, Ss	0	2	1
	R:H−:F/S:Du, Ma, Rf, Rh, Ss, Sc	3	0	0
	R:H18:A:Du, Ma, Rf, Rh, Ss, Sc	1	0	0
7	O42:H19:F/S:Du, Ma, Rf, Rh, Sc	1	0	68
	O42:H19:A:Du, Ma, Rf, Rh, Sc	7	0	0
	O42:H19:F/S:Du, Ma, Rh, Sc	12	0	0
	O42:H19:A:Du, Ma, Rh, Sc	1	0	0
	O42:H7:F/S:Du, Ma, Rf, Rh, Ss	1	0	0
	O20:H19:F/S:Du, Ma, Rf, Rh, Sc	0	0	1
	O96:H19:A:Du, Ma, Rf, Rh, Sc	1	0	0
	R:H19:F/S:Du, Ma, Rf, Rh, Sc	0	0	1
	R:H7:F/S:Du, Ma, Rf, Rh, Ss	6	0	0
17	O71:H11:F/S:Ma, Rh	7	0	42
	O81:H−:F/S:Ma, Rh, Ss	2	5	0
	O81:H−:F/S:Ma, Sc	3	0	1
	O81:H11:F/S:Ma, Rh	0	0	1
	O147:H−:F/S:Ma, Rh, Ss	0	1	0
	R:H−:F/S:Ma, Sc	0	0	2

Table 7. *Complex variation of selected strains isolated during a ten-day period*

Date	Patient	Strain
8 May 1972	24 mother	O9:H38:F/S:Du, Ma, Rf, Rh
13 May 1972	24 mother	R:H52:F/S:Du, Ma, Rf, Rh, Sc
13/15 May 1972	24 mother	O46:H52:F/S:Du, Ma, Rf, Rh, Sc
14 May 1972	23 mother	O46:H52:F/S:Du, Ma, Rf, Sc
14 May 1972	23 mother	R:H52:F/S:Ma
14/16 May 1972	26 mother	O46:H38:F/S:Du, Ma, Rf, Sc
15 May 1972	30 mother	R:H52:F/S:Du, Ma, Rh, Sc
15 May 1972	24 mother	R:H52:F/S:Du, Ma, Rf, Rh, Ss, Sc
15 May 1972	29 mother	R:H52:F/S:Du, Ma, Rf, Rh
16 May 1972	27 mother	O46:H52:F/S:Du, Ma, Rf, Sc
18 May 1972	30 mother	O46:H52:F/S:Du, Ma, Sc
18 May 1972	30 mother	R:H52:F/S:Du, Ma, Sc

strains possessing identical markers indicating classical cross-infection (Bettelheim *et al.* 1974).

To date, it has been assumed from epidemiological investigation of *E. coli* spread that the antigenic markers exhibit only limited variations, e.g. O–R and H+/H−. There has been one report of serial study of serological typing of organisms causing chronic urinary infection which showed complex variations of sero- and biotypes

(Bettelheim & Taylor, 1969). The present study suggests that this phenomenon may be more widespread than previously assumed. The genetic basis for this is under investigation.

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Use of single radial immunodiffusion test for serological studies in volunteers inoculated with live attenuated influenza virus

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SUMMARY

Pre- and post-vaccination serum samples from 278 volunteers, who were given live influenza vaccines, were tested by haemagglutination inhibition (HI) and single radial immunodiffusion tests(SRDT) for antibody to influenza A/Hong Kong/1/68 (H3N2) virus envelope antigens. Those with high antibody titres detected in both tests were less frequently infected, and 85% of the 159 infected showed rises by HI and 70% by SRDT. Similarly, 69 pairs were tested for antibody to Hong Kong (N2) neuraminidase by neuraminidase inhibition test (NI) and by SRD tests. Those with high titres in both tests resisted infection and those who were infected showed a rise in antibody detected both by NI and SRD tests. In general, SRDT was less sensitive than HI and NI in detecting antibody and antibody rises, but in some volunteers it did detect antibody rises which were not detected by conventional tests. Because of its simplicity and speed it appeared to be of use in evaluating such vaccines.

INTRODUCTION

The measurement of circulating antibodies against antigens of the influenza virus is important in the study of influenza epidemiology and in the assessment of the efficacy of vaccines. Antibodies directed against the envelope proteins of haemagglutinin and neuraminidase are conventionally measured using some version of the haemagglutination-inhibition (HI) test (Hirst, 1942) and the neuraminidase-inhibition (NI) test (Webster & Laver, 1967). Complement-fixation tests (CFT) are used to detect antibody to the internal ribonucleoprotein antigen. The technique of single radial immunodiffusion has been applied to measurement of antibodies and antigens (Mancini, Carbonara & Heremans, 1965; Vergani, Stabilini & Agostini, 1967). Schild, Henry-Aymard & Pereira (1972) described the use of a single radial immunodiffusion test (SRDT) for influenza antibodies. The test is more convenient and rapid than the HI or NI tests or CFT (Schild *et al.* 1972). In the present study the SRDT is compared with the conventional tests in the

study of sera from volunteers inoculated with live influenza virus. The methods are compared for sensitivity in detecting initial serum antibody levels, in predicting succeptibility to infection and in detecting antibody rises after infection.

MATERIALS AND METHODS

Volunteers

The sera studied were from 278 volunteers who had taken part in a number of trials at the Common Cold Unit, Harvard Hospital, Salisbury, between 1968 and 1970. Standard methods of isolation of volunteers, nasal inoculation of virus, the taking of nasal washings and clinical observation were used (Tyrrell, 1963; Beare, Bynoe & Tyrrell, 1968).

Inoculation of volunteers

After the initial blood sample had been taken, each volunteer was given live influenza virus by intranasal inoculation. The virus used in each case was influenza A/Hong Kong/1/68 (H3N2) or a recombinant having the same surface antigens (H3N2). In most trials the dose used was $10^{5\cdot5}$ EID50. In some $10^{6\cdot5}$ EID50 was used. Nasal washings were taken on the 3rd and 4th days after inoculation and virus isolation from washings was attempted.

Serology

Second serum samples were obtained from volunteers 2–3 weeks after inoculation. All sera were stored at $-20°$ C. HI tests (Tyrrell, Peto & King, 1967) were carried out on all of the 278 pairs of sera, and NI tests (Aymard-Henry *et al.* 1973) on 69 pairs. All were tested by single radial diffusion in plates containing X31 virus (Kilbourne, 1969) containing surface antigens (H3 and N2) identical with those of A/Hong Kong/1/68 virus. Those sera on which the NI test was performed were also tested by radial diffusion in plates containing X15 HK virus (surface antigens HEq1N2).

Single radial diffusion test

The immunoplates employed (Schild *et al.* 1972) consist of agarose gel incorporating a homogeneous suspension of intact, purified influenza virus. Test sera (5 μl. volumes) are placed in 2 mm. diameter wells cut in the agar, and the presence of antibody to viral surface antigens is detected by the appearance of a zone of opalescence surrounding the wells (see Plate 1).

In plates containing A/Hong Kong/1/68 (H3N2) virus (X31 strain), opalescence may be due to either anti-haemagglutinin or anti-neuraminidase or both. In plates containing the recombinant strain A/Equi1/Prague/56(HEq1)–A/Hong Kong/1/68 (N2) only anti-neuraminidase antibody is detected as anti-HEq1 antibodies are not found in human sera.

In this study the size of reaction is recorded as the diameter of the opalescent zone. The minimum zone diameter considered as significant was taken as 2·2 mm. A significant difference between two readings is one of 10% or greater (Schild, Berryman, Pereira & Henry-Aymard, 1973).

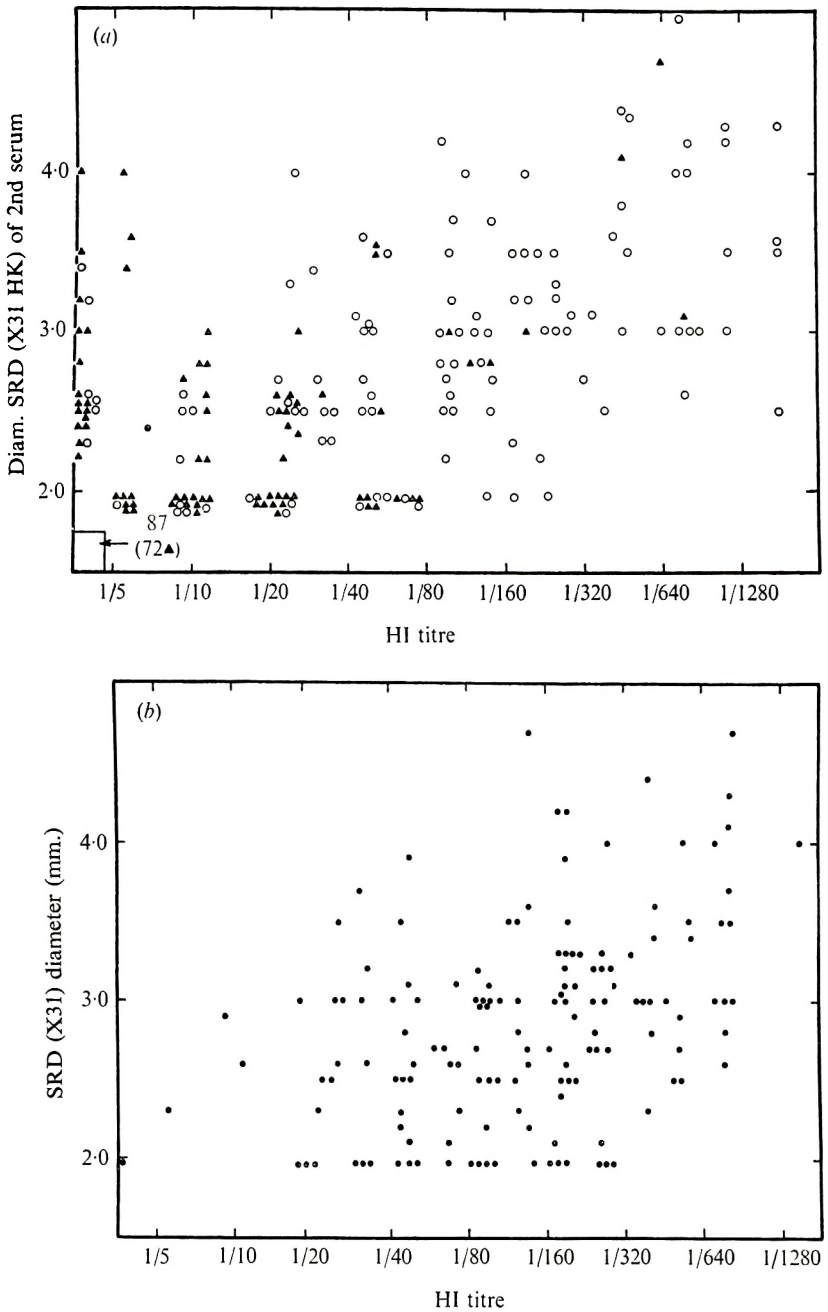


Fig. 1. (*a*) The results of titrating the first specimens of sera from subjects who did and did not become infected when subsequently inoculated with live influenza vaccine. Both HI and SRD tests were done with viruses of the Hong Kong (H3N2) serotype and the challenge viruses belonged to this serotype also. There is a correlation between the results of the two tests ($r = 0{\cdot}6437$, $n = 277$, $P < 0{\cdot}001$). ▲, Subjects who became infected: ○, subjects who did not become infected. (*b*) The results of titrating the second specimens of sera from subjects who became infected. There is again a positive correlation (for all second sera $r = 0{\cdot}5128$, $n = 276$, $P < 0{\cdot}001$).

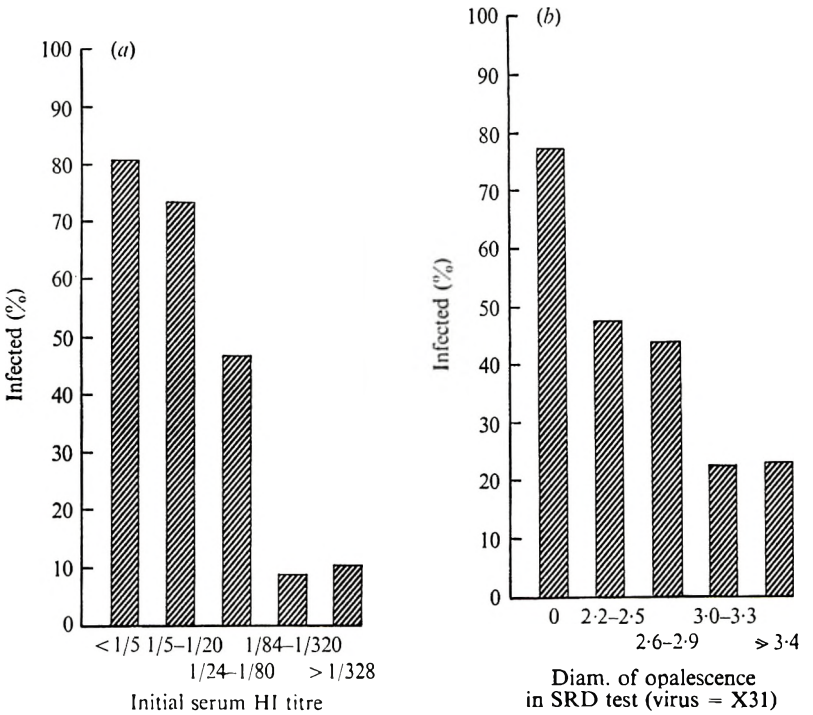


Fig. 2. Percentage of volunteers who were infected in relation to their serum antibodies before infection: (*a*) as measured by HI and (*b*) by SRD.

Evidence for infection of volunteers

For the purposes of this study, a subject was judged to have been infected by the inoculated virus if one or both of the following criteria were fulfilled: (*a*) virus isolated from nasal washings on 3rd and 4th days; (*b*) a fourfold or greater rise in HI titre 2–3 weeks after inoculation.

RESULTS

Antibody measurement

The correlation between HI titres and the diameter of the opalescent zone on X31 plates is shown in Fig. 1(*a*) and (*b*). Fig. 1(*a*) shows the results for initial (pre-inoculation) sera. There is a positive correlation between the results obtained by the two tests; but of the 277 sera, there were 49 (18 %) in which antibody was detected by the HI test and not by SRD and 22 (8 %) in which SRDT but not HI indicated the presence of antibody. Fig. 1(*b*) represents post-vaccination sera: only sera from those volunteers who were infected by the inoculated virus, as judged by the above criteria, were included. Again a positive correlation was found. In 21 of the 152 sera (13·8 %) no antibody was detected by SRDT.

Prediction of susceptibility

In Fig. 1(*a*), which shows all initial sera tested by the two methods, those subjects who were infected by the inoculated live virus are represented by filled

Table 1. *Rising antibody titres detected by HI and SRDT, and rate of virus isolation from the respiratory tract*

No. of paired sera with single radial diffusion (virus = X31) showing	No. of paired sera with HI titres showing		
	No rise	Significant rise	Totals
No rise	129 (12:9 %)*	36 (18:50 %)	165 (30:18 %)
Significant rise	12 (4:33 %)	99 (75:76 %)	111 (79:71 %)
Totals	141 (16:11 %)	135 (93:69 %)	276 (109:39 %)

* Figures in parentheses show the number and the percentage of subjects in each group yielding virus from nasal washings on day 3 or 4 after inoculation.

triangles; those who did not become infected, though inoculated with live virus, are represented as open circles. It is clear that the greatest incidence of infection is amongst those with low antibody measured by either test. There are some subjects who succumb to infection despite high initial antibody levels, whether measured by the HI or SRDT. The proportion of 'susceptible' people in groups with various initial antibody titres is shown in Fig. 2(*a*) and (*b*). For both tests, the proportion of subjects becoming infected decreases with increasing initial antibody titre.

Antibody responses to infection

Table 1 represents the changes in antibody titres in all pairs of sera as measured by HI and by SRDT using X31 immunoplates. Figures in parentheses indicate the number of subjects in each category from whom virus was isolated in nasal washings taken on the 3rd or 4th day after inoculation. In the majority of subjects, virus isolation is accompanied by a significant rise of antibody measured by both methods. There were 12 who were infected, as judged by virus isolation, in whom no serological evidence for infection appeared by either test. In a further 36 there was a fourfold or greater rise in HI titre with no rise shown in the SRDT. In 12 (4 of whom yielded virus from nasal washings) a rise of antibody titre appeared in the SRDT with no change in HI titre. If the criteria of infection are enlarged to include (*a*) virus isolation, (*b*) fourfold or greater rise of HI titre and (*c*) significant rise on SRDT, then from Table 1, the number infected was

$$12 + 36 + 12 + 99 = 159.$$

Of these, 135 (85 %) would have been revealed by the HI test alone and 111 (70 %) by the SRTD alone, using X31 plates.

Antibody changes in a random sample of the paired sera tested are shown graphically in Fig. 3. Most pairs are represented as an oblique line, indicating a rise of antibody titre by both tests. For some the line is vertical, indicating a rise in HI titre with no change in SRD. Three pairs are represented by a horizontal line, in which there is an increase in the SRDT but no change of HI titre. Two of these pairs also showed an increase on the X15 HK plate, suggesting that the antibody rise may be due to an increase in anti-neuraminidase antibody

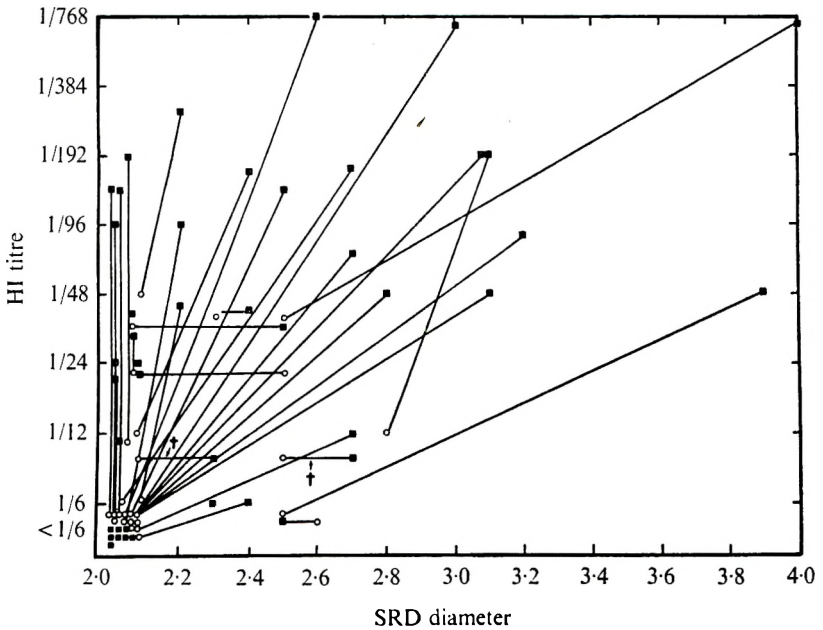


Fig. 3. Antibodies of volunteers before and after inoculation as measured against Hong Kong by HI and SRD. The first and second serum values of each subject are joined by a line. In those indicated thus† a rise of antineuraminidase antibody was detected by SRDT using X15. ○, Before vaccination; ■, after vaccination.

Table 2. *Rising antibody titres detected by NI and SRDT, and rate of virus isolation from the respiratory tract*

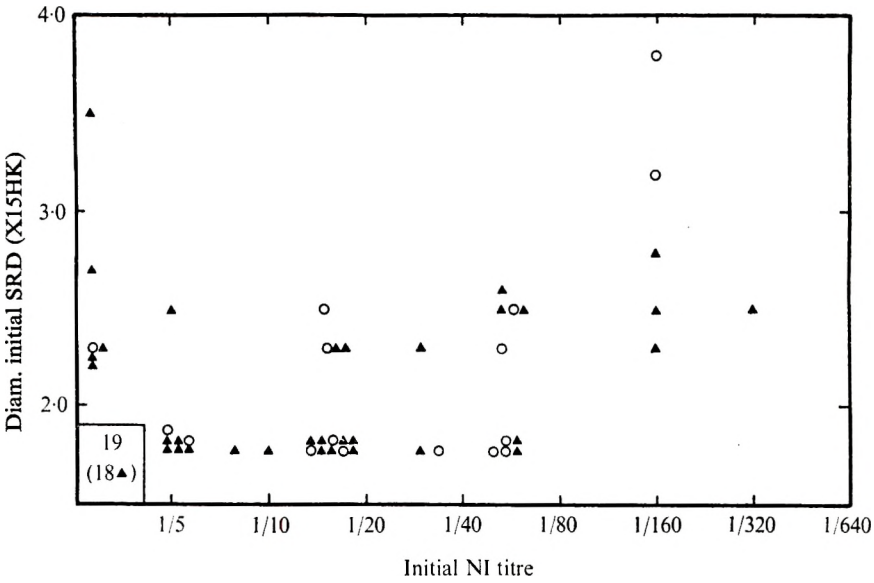
No. of paired sera with single radial diffusion (virus = X15HK) showing	No. of paired sera with NI titres showing: No rise	Significant rise	Totals
No rise	28 (8:29 %)	10 (7:70 %)	38 (15:40 %)
Significant rise	8 (7:87 %)	23 (20:87 %)	31 (27:87 %)
Totals	36 (15:42 %)	33 (27:82 %)	69 (42:61 %)

* Figures in parentheses show the number and the percentage of subjects in each group yielding virus from nasal washings on day 3 or 4 after inoculation.

only: this would be measured as a rise on the X31 plate, but might not be detected by HI tests.

Anti-neuraminidase antibody

The neuraminidase inhibition (NI) test was carried out on a random selection of 69 pairs of the total number of paired sera available. These 69 pairs of sera were also tested by SRD in plates containing the virus X15 HK(HEq1N2), to detect anti-neuraminidase antibodies. The correlation between NI and SRDT is shown in Fig. 4(*a*) and (*b*). The correlation is better for the post-infection sera; in both cases there are more sera in which antibody is detected by NI test alone than by SRDT alone. In Table 2 it can be seen that in most subjects who were



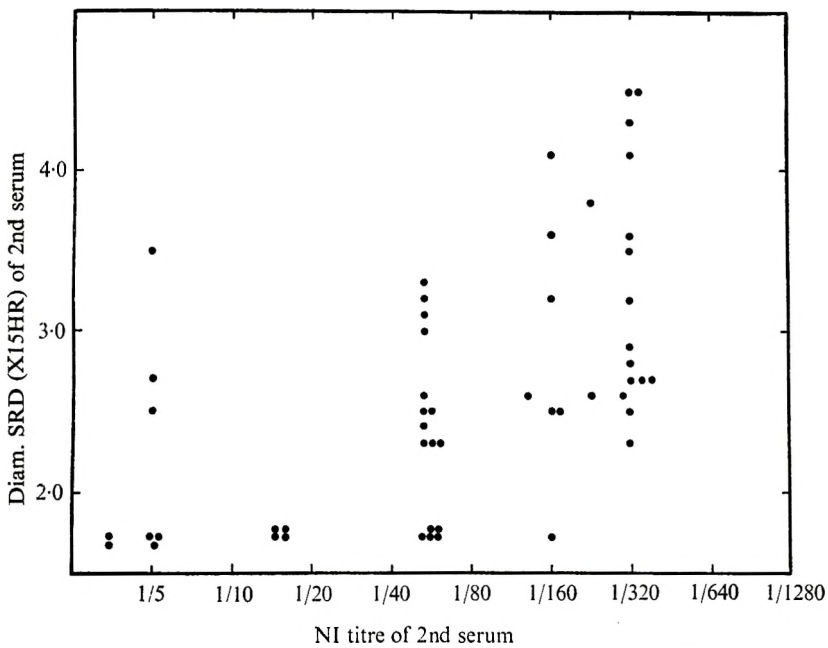


Fig. 4. (*a*) The results of titrating the first specimens of serum from subjects who did and did not become infected when subsequently inoculated with live influenza vaccine. NI and SRD tests were done with the N2 serotype. ▲, Infected; ○, Uninfected. (*b*) The results of titrating the second specimens of serum from subjects who became infected.

infected as judged by virus isolation, there is a rise of anti-neuraminidase antibody. In some of these the rise is detected only by NI test, and in a similar number by SRDT only. In a few there is no rise in either test.

DISCUSSION

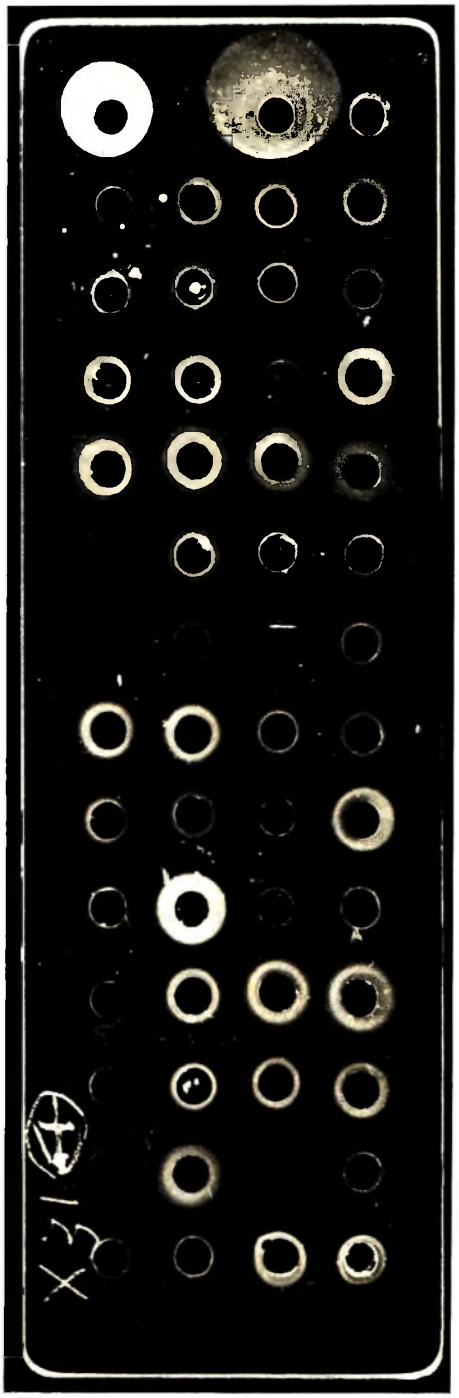
As live influenza vaccines are developed serological studies on large numbers of people will be necessary for their evaluation and simple and precise methods are therefore desirable. The single radial diffusion test has several advantages over conventional tests in serological studies for this purpose (Schild *et al.* 1973). It is quick and easy to perform. If necessary, large numbers of sera can be tested in field conditions, and plates can be posted for subsequent reading. Fifty-six sera can be tested on a single plate measuring 1 in. × 3 in. Whole fresh blood may be used if necessary; very small volumes are sufficient, so that finger-prick may be used instead of venepuncture. Since non-specific inhibitors of haemagglutination do not react in SRDT no pretreatment of samples is needed. Plates can be photographed to provide a permanent record. Antibodies against various constituents of the influenza virus can be measured if appropriate recombinant or disrupted virus is incorporated in the agarose gel.

A disadvantage of the method is that greater quantities of virus are needed for the test plates than would be needed for HI tests on the same number of sera, but this problem is minimized by the availability of high yielding recombinant strains of influenza such as X31. As a method of detecting anti-neuraminidase the method offers considerable advantages because of the complexity of the enzyme-inhibition test.

The results of this study of the sera of volunteers given live virus vaccines indicate that SRDT give information which is similar to that which can be obtained from HI and NI tests, although the SRDT have been less sensitive. There is a lower rate of detection of antibody in initial serum samples, and of antibody rises in paired sera when the SRDT is used. The correlation between antibody level and resistance to infection is better when antibody is measured by the HI test than by SRD (Fig. 3*a*, *b*).

The greatest amount of information is obtained when both conventional tests and SRDT are used. However, the SRDT alone provide information on antibody status, susceptibility and serological response which could be of considerable value in the study of live vaccines. The disadvantages of the apparent lower sensitivity of the test must be weighed against the advantages of simplicity and speed in the assessment of large numbers of sera. The sensitivity would be less important in comparisons between different vaccines, and S. R. Mostow, G. C. Schild and W. R. Dowdle (unpublished) have used the test to evaluate the antibody responses following killed influenza vaccines and natural infection and have found the SRDT method to be more sensitive than conventional HI and CF tests in detecting antibody rises. Recent unpublished studies show that a test using 10 μl. of serum and 3 mm. wells is more sensitive than HI.

The relatively poor correlation (Table 1) between antibody titres measured by



SRD and the results obtained with the HI test suggest that the two tests may be measuring different antibodies. In this connexion it is relevant that J. L. Virelizier, G. C. Schild, R. Postelthwaite & A. C. Allison (in preparation) have shown that antibodies against different antigen determinants of the haemagglutinin molecule can be detected and identified by SRDT. It seems likely from these studies that SRDT is capable of detecting antibody against a wider range of the antigenic determinants of the haemagglutinin molecule than is the HI test. In particular, a modification of the SRDT, involving antibody adsorption procedures, enabled the independent assay of antibody to strain-specific antigenic determinants of the haemagglutinin and cross-reactive determinants (shared between members of a single haemagglutinin subtype) and evidence was obtained suggesting that strain-specific antibody is likely to be more closely related to immunity than cross-reactive antibody. It will be of interest to continue our studies on antibody responses to various types of influenza vaccines using strain-specific and cross-reactive antibody assays as measures of the immune response.

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EXPLANATION OF PLATE

A typical plate showing the zones of opalescence, the diameters of which were measured.

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The provision of bacteriologically safe infant feeds in hospitals

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SUMMARY

Infant feeds, to be safe, must be free from potentially pathogenic organisms but not necessarily sterile. In-bottle terminal heating is the preferred means of producing such feeds and the advantages and disadvantages of high and low pressure heating methods, including the effect upon the food value of the feeds, are discussed. The safety of a low pressure method in use in Princess Alexandra Hospital, Harlow is described. The choice of method of provision of safe feeds; terminal heating in a central milk kitchen or obtaining a commercial supply should be decided on economic grounds. Hospitals using such commercial supplies, however, should make provision for training mothers in the hypochlorite method of disinfection of bottles and teats and also ensure that their trainee nurses and midwives still receive adequate instruction in the hygiene of feed preparation.

INTRODUCTION

It has long been recognized that contamination of infant feeds plays an important part in the spread of epidemic gastro-enteritis in nurseries for the new-born (Lembcke, 1941; Cumming, 1949) and that means to control this disease should include encouragement of breast feeding, as gastro-enteritis is relatively rare in naturally fed babies (Hinton & McGregor, 1958; Bullen & Willis, 1971), and the provision of a central milk kitchen where safe feeds can be prepared to high bacteriological standards. These measures assume even greater importance now that the value of antibiotics in this disease is called in question (British Medical Journal, 1972).

The provision of safe feeds may be achieved by a cold, chemical method, which is synonymous with hypochlorite disinfection of bottles, teats and equipment (Farquhar, Gould & Schutt, 1965) and has the advantage of cheapness but has been criticized (Ayliffe, Collins & Pettit, 1970) for inadequate safety. Alternatively, prepared and bottled feeds with the teats already in place may be heat treated. This may be either a high or low pressure process and each has its advocates. In-bottle retort sterilized feeds are now available commercially at a competitive price and provide a safe alternative to the hospital processed product.

Terminal heating processes

The American Hospital Association Manual 'Procedures and layout for the Infant Formula Room' describes all aspects of terminal heating and considers both high and low pressure methods to be safe and satisfactory. Both methods are described by Perkins (1956) who gives an excellent account of the techniques,

Table 1. *Terminal heating processes compared*

High pressure method	*Low pressure method*
Bacteriologically safe	Bacteriologically safe
Time-temperature relationship 110–111° C. for 10 min.	Time–temperature relationship 90–100° C. for 5–30 min. (Various authors)
Total process time including slow exhaust of pressure. 30–40 min. (Perkins, 1956)	Total process time – no exhaust of pressure. 30–40 min. (Perkins, 1956)
Chemical changes produced in milk by coagulation of protein, caramelization and slight destruction of vitamins	Minimal chemical changes in milk. Slight destruction of vitamins
Over exposure produces exaggeration of physical and chemical changes	Moderate over exposure produces minimal increase in physical and chemical changes, except caramelization (Strachan, 1964)
Careful mechanical control necessary to prevent boiling, clogging of nipples, etc.	Minimal mechanical control needed. No boiling
No refrigeration of end product (Tomlin *et al.*, 1966)	Rapid cooling and refrigeration of end product essential
No pre-sterilization of bottles and teats (disputed by some authors). Minimal aseptic care needed in preparation room	Pre-sterilization of bottles and teats very advisable. Reasonable aseptic care needed in preparation room
Bottles and equipment must be clean	Bottles and equipment must be clean
Bottles and teats need not be decontaminated before return to kitchen (Tomlin *et al.*, 1966)	Decontamination of bottles and teats not essential provided they are returned to a washing room separate from the preparation room

organization and layout of facilities for each. He considers the low pressure method the more advantageous. A high pressure method is recommended by Bolton (1966) a hospital engineer, who also gives details of layouts for varying sizes of units, and is strongly advocated by Tomlin, Tomkin & Dorward (1966) although they differ from Bolton on a number of points.

The low pressure method is described by Rourke (1947*b*), by Strachan (1964) and by Hughes, Darmady & Drewett (1966), who also examined a commerical sterilizer-cooler. Both methods were thoroughly investigated by a team of workers in Illinois (Smith, Finley, Wright & Louder, 1948; Finley, Smith & Louder, 1948). These American workers regard exposure of feeds to steam at either 100° C. (212° F.) for 15 min. (low pressure) or 110° C. (230° F.) for 10 min. (high pressure) as safe and adequate. In one commercial process the vacuum capped bottles are held at 121° C. (250° F.) under water for 8 min.

There is general agreement with the Illinois workers' recommendation for the high pressure process but Perkins (1956) recommends 100° C. (212° F.) for 30 min. for the low pressure method while Hughes *et al.* (1966) feel the makers' recommended holding time of their commerical apparatus, 25 min. at 100° C. (212° F.) could be safely reduced to 10 min., twice the adequate period found in their experiments. Rourke (1947*a*) found 5 min. exposure provided a high percentage of sterile feeds.

A comparison of each system is summarized in Table 1, adapted from Perkins.

The appearance on the British market of commercial retort-sterilized feeds necessitates a re-appraisal by hospitals of the processes in use and the situation in Princess Alexandra Hospital, Harlow, can be taken as an example

METHODS AND RESULTS

Process in use in Princess Alexandra Hospital, Harlow, at present

In Harlow we have been using a low pressure method in a central milk kitchen for over 6 years with success. This process utilizes a short holding period controlled by a thermocouple inserted into a bottle which indicates the feed temperature on a dial mounted on the autoclave. When this indicates a temperature of 72·5° C. the flow of steam is cut off after 3 min. and the load immediately removed from the chamber. In practice the temperature of the feeds just reaches 100° C. when heating is discontinued. This is essentially a flash pasteurization process (71·5° C. for 15 sec.) with a prolonged holding time as an added safety factor. The feeds require air cooling, for 30 min., and subsequent refrigeration. The apparatus in use is a downward displacement autoclave with the addition of a free-steam facility and the thermocouple described above.

The cycles provided are:

1. Sterilization of bottles and teats – 121° C. for 15 min. – automatically controlled.
2. Free steam – manually controlled.

The refrigerator is a heavy duty model as advised by Perkins (1956).

The filled bottles are, of course, fitted with nipples before processing. The nipples were originally protected by paper bags held in place by rubber bands but latterly loosely fitting metal foil caps were found to be preferable in that they did not tend to stick to the teats while still allowing steam to reach them.

Previous testing had shown that after processing a proportion of feeds (approximately 14%) still contained aerobic spore-bearing bacilli, samples being taken from all parts of the autoclave chamber. As the need for the provision of completely bacteria-free feeds is debatable, tests were carried out on feeds deliberately contaminated with the following pathogens, *Staphylococcus aureus* from a breast abscess, yeasts from a case of infantile thrush, *Eschericia coli* from an infant's stool, Beta haemolytic streptococci from an adult sore throat and in addition, another feed was contaminated with adult stool. The results are summarized in Table 2. Aerobic spore-bearers were isolated from each sample but it was concluded that the process was capable of killing vegetative organisms including yeasts. It was also established that feeds would remain sweet and unspoiled in the refrigerator for a period of at least 12 weeks, although it was never intended that they would be kept for more than 2 days.

As it is realized that careful technique and maintenance of the apparatus by well-trained, conscientious staff is the only real safeguard of the quality of the product, 'in-use' bacteriological tests on the feeds are routinely carried out at only two-weekly intervals.

Table 2. *Treatment of artificially contaminated feeds*

Organism	Bacterial count before process	Bacterial count 24 h. after process	Bacterial count 3 weeks after process
Yeast	83,000/ml.	Nil	Nil
Staph. aureus	More than 250,000/ml.	Nil	Nil
Esch. coli	More than 250,000/ml.	Nil	Nil
Beta haemolytic streptocci	More than 250,000/ml.	Nil	Nil
Stool (no *C. welchii*)	—	Nil	Nil

Bacteriological control

Pour plate counts using Oxoid Blood Agar base, are made on feeds taken at random from the refrigerator 24 hours after processing. The mean count of two or three 1·0 ml. samples of neat feed is recorded. The teats are removed aseptically and immersed in honey pots of Oxoid broth for qualitative culture only. The pour plates are incubated at 37° C. for 24 hr. and at room temperature for a further 24 hr. before the counts are made, while the broths containing teats are incubated at 37° C. for 24 hr. and then subcultured on blood agar for another 24 hr. at 37° C. before reading.

The results from the feeds are very satisfactory. In no instance has an organism, other than an aerobic spore-bearing bacillus, been isolated and apart from a short period when the thermocouple was found to be faulty, counts have always been less than ten organisms per millilitre, only seven percent being above five organisms per millilitre. According to Perkins (1956) feeds containing less than 25 organisms per millilitre, tested after 24 hr. refrigeration, are acceptable; this is the standard also adopted by the American Hospital Association (1965). Lowe (1947) used two standards: (*a*) absence of coliform organisms and (*b*) less than 50 organisms per millilitre of feed. Cumming (1949) suggested 500 organisms per millilitre of feed after 24 hr. refrigeration. It is obvious that the process in use here meets the strictest of these requirements and equals the finding of Smith *et al.* (1948) that the low pressure method can destroy vegetative organisms and reduce the count of spore-bearers to less than ten organisms per millilitre.

The fault in the thermocouple already mentioned was associated with feed counts rising suddenly to 31, 36, 50 and over 100 organisms per millilitre. The counts fell to the usual level as soon as the fault was remedied.

The teats have less frequently been found to be sterile, although this possibly reflects the difficulty in their aseptic removal for testing, 33 % being sterile and 67 % growing either aerobic sporers or *Staphylococcus albus* (coagulase negative). No quantitative testing methods have been used but a method has been described by Rourke (1947*a*) who studied the effect of the low pressure method and found 10 min. exposure was satisfactory. Unfortunately he did not record any results for teat tests after 5 min. exposure as he did for the actual feeds.

On three occasions only have our teat cultures revealed pathogens or potential pathogens, *Pseudomonas* spp. once and *Staphylococcus aureus* (coagulase positive) twice. These were isolated findings not found on testing further teats and not

associated with any upset in the babies. They did, however, stimulate a re-testing of the ability of the process to kill *Staph. aureus* and tests to show that *Pseudomonas pyocyanea* was killed in artificially contaminated milk.

It can be concluded that the Harlow process is safe and has been proved to be so by continuous use for over 6 years. Further factors, however, require consideration in the choice of method of providing safe feeds. Some of these will now be discussed.

DISCUSSION

Retention of nutrient value of feeds

Some paediatricians express misgivings about the destruction of vitamins in the heat processing of infant feeds. This question was studied by Hodson (1949) who concluded that the low pressure method conserved 95% of ascorbic acid, 91% of thiamine and 100% of lysine. The high pressure method conserved the same amounts of thiamine and lysine and even greater amounts of ascorbic acid, 97%. In view of these small losses of heat-labile nutrients there is little reason to fear significant loss of more heat-stable constituents. Excess vitamins could be added to the feeds to restore any postulated loss should it be necessary to keep babies in hospital for longer than the usual few days post partum when any loss of vitamin intake must be negligible.

Training of mothers and nurses in bottle feeding

One drawback to the central milk kitchen or the use of commercial supplies is that provision must be made to train mothers to prepare safe feeds once they have returned home. This involves the provision of a training room.

Home standards of hygiene of infant feeding utensils have been found deficient in surveys carried out in Britain. Gatherer & Wood (1966) found only 69% of bottles and 46% of teats 'satisfactory' in homes visited in Reading. Anderson & Gatherer (1970) found 'less than two-thirds of bottles and just over half the teats satisfactory' in four separate areas of the U.K. Wright (1951) testing feeds rather than bottles found home prepared feeds unsatisfactory even making allowance for the time lag between preparation and testing. Only 13% of her sample had zero counts and 30% contained over one million organisms per millilitre in feeds prepared by mothers attending out-patient departments, although those attending infant welfare clinics produced more satisfactory results. The hospital prepared feeds studied by this author also gave few grounds for satisfaction.

The first two studies stress that mothers do better with hypochlorite methods than heat. Graham (1961) studying teats, also states that dry sterilization (pressure cooking) preserves the rubber longer than wet procedures but hypochlorite solution causes less deterioration than boiling. Whether this point is still valid for Latex teats is doubtful but the conclusion to be drawn is that mothers are more likely to produce safe feeds at home if they are taught how to do so before leaving hospital and that they should be taught the hypochlorite method.

It is obvious also that provision for training of nurses and midwives in the hygiene of feed preparation must continue.

Economics

As hospital methods of heat treatment are safe and advantageous, the decision whether to provide a central milk kitchen or to use a commercial source of pre-sterilized baby feeds must be made on purely economic grounds. The economics of milk kitchen procedures require attention by hospital administrators. This problem has been little studied but two publications are relevant. Schenkweiler *et al.* (1960) concluded after a survey of methods in six American hospitals that a medium-sized hospital can prepare a bottle of infant feed as cheaply as it can be purchased from a service company but efficient production and handling methods and low cost labour for work not requiring trained nurses must be used. Hurst (1968) in Britain concluded that the majority of hospitals could usefully look into their present milk kitchens to ascertain whether the best use is being made of resources. He also found the greatest possible economy to lie in labour costs.

The Harlow process was costed in 1970 at 9·4d (3·9p) per feed which was only marginally less than a commercial preparation at that time available. Since then there have been several increases in labour costs and in 1973 our product probably costs marginally more than a commercially supplied feed as many costs are hidden and difficult to evaluate such as maintenance of equipment, electricity, refrigeration and cost of delivery to the consumers. In addition the autoclave is becoming increasingly difficult to maintain owing to obsolescence, nursing staff are increasingly difficult to find and must be deployed as productively as possible and the maternity department is shortly to be greatly increased in size. As a result of all these factors a decision to change to a commercial product in the near future appears to be correct.

Hospitals possessing milk kitchens must make this decision in the light of their own circumstances. Hospitals without central milk kitchens should consider well before tying up large sums of capital in the provision of equipment.

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Variation of serotype in strains of *Bordetella pertussis*

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SUMMARY

The four main serotypes of *Bordetella pertussis* (1, 2, 3; 1, 2; 1, 3; 1) undergo spontaneous variation involving loss or gain of antigen 2 or antigen 3. By serial subculture from single colonies on charcoal-blood-agar medium, we have detected loss-mutations from type 1, 2, 3 to 1, 2 or 1, 3, and from type 1, 2 to type 1. Likewise we have found gain-mutations from type 1 to 1, 2 or 1, 3, and from 1, 2 to 1, 2, 3.

These mutations apparently occur with a high frequency in some strains. Other strains have a lower mutation-rate and are more stable antigenically. We have not detected, by this method, either gain- or loss-mutations from the type 1, 3 strains that we have tested.

These findings offer an explanation for the changes in serotype that occur during the course of a pertussis infection in the child and in the marmoset. They also constitute a warning on the possible antigenic instability of laboratory strains, especially relevant in the production, absorption and testing of diagnostic antisera and in the preparation of pertussis vaccine.

INTRODUCTION

It was suggested by Cameron (1967) that *Bordetella pertussis* may undergo a stepwise loss of heat-labile antigens, converting a parent of type 1, 2, 3 to a degraded strain of type 1. This suggestion is supported by our previous findings in the child (Preston & Stanbridge, 1972) and in the marmoset (Stanbridge & Preston, 1974), in which we showed that the parent (1, 2, 3) and the intermediates (1, 2; 1, 3) could each establish infection and could change to a different serotype during the course of the infection, whereas type 1 organisms were found only at a late stage of the infection and, even then, did not constitute the predominant serotype.

The object of our investigation was to obtain some indication of the frequency with which such changes in serotype may occur spontaneously *in vitro*, as this may be of importance for the antigenic stability of laboratory strains used in the preparation of diagnostic antisera and of pertussis vaccine.

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MATERIALS AND METHODS

Strains of Bordetella pertussis

Four of our strains had been isolated from children in England: three in 1967 (M/S23 in Manchester, SF/S8 in Sheffield, 41633 in Coventry) and one in 1970 (M21356 in Manchester). All were initially mixed cultures of more than one serotype (Tables 1 and 2). The fifth strain was the Kendrick challenge strain, W.18–323, which in our laboratory is a type 1 strain (Preston, 1966).

Serotyping of pertussis strains

Full details have been described previously (Preston, 1970).

Typing and serial subculture of single colonies

Each strain was cultured on a plate of charcoal-blood-agar (Oxoid, 1965) so as to obtain, after 3–4 days at 35–36° C., confluent growth suitable for serotyping, and also well-separated colonies for subculture. Individual colonies of *Bord. pertussis*, of this age, are too small for accurate serotyping, whilst older colonies tend to give auto-agglutinable suspensions. Serotyping of single colonies was, therefore, determined retrospectively by testing the confluent growth of the next subculture.

On each occasion, about eight single colonies were subcultured, each on a separate plate, and single colonies from one such plate were used for the next subcultures. Whenever a change of serotype was detected on one of the eight plates, several single colonies were subcultured from this plate as well as from a plate with the original serotype.

RESULTS

Initially, four of the five strains recorded in Tables 1 and 2 were mixed cultures of two or three different serotypes: not all the colonies of the first plate culture were of the same serotype. But, with all five strains, single-colony subculture usually yielded a pure growth of the same serotype as the parent colony. Occasionally, whereas the majority of colonies tested were of the same serotype as the parent, a single colony of a different serotype appeared, and this in turn yielded pure subcultures of the new serotype. The emergence of these occasional mutant colonies, in an otherwise pure population, is strong evidence to suggest that each colony was usually derived from a single cell, for mixed populations were rarely encountered after the first two subcultures.

Ignoring, then, any changes of serotype detected in less than three serial single-colony subcultures, there is evidence of both loss- and gain-mutation. In Table 1, all three strains show loss of antigen 2 from type 1, 2, 3. Strain M/S23 shows also loss of antigen 3, changing 1, 2, 3 to 1, 2, and subsequent loss of antigen 2, changing 1, 2 to type 1. The only loss-mutation, concerning antigens 2 and 3, that was not detected was the change from 1, 3 to type 1. On two occasions, with strain SF/S8, type 1 growth was found in subculture from a type 1, 3 parent, but this was only a phenotypic suppression of antigen 3, the cause of which is not known (Preston, 1970). On both occasions, further subculture reverted to a pure growth of type 1, 3.

Table 1. *Variations of serotype involving* loss *of antigen* 2 *or antigen* 3

Strain	Serotype of original culture	Serotypes of colonies tested	Serial no. of subculture* . . . 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23–27
			No. of colonies tested on each subculture (tabulated according to serotype)																						
M/S23	1, 2, (3)	1, 3, . . .				2	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8					
		1, 2, 3, . . .	1	→ 1	→ 4	6	→ 8	→ 8	→ 8	→ 8	→ 7	→ 8	→ 8	→ 7											
		1, 3, . . .													1	→ 4	→ 4	→ 4							
		1, 2, . . .	6								1	→ 8	→ 8	→ 8	→ 8	→ 7									
		1, . . .	1														1	→ 8	→ 8	→ 8					
M21356	1, (2), 3	1, 2, . . .												1†											
		1, 3, . . .		2	→ 12		1														1	→ 8	→ 8	→ 8	
		1, 2, 3, . . .	1	4	→ 9	→ 6	8	→ 1	→ 1	→ 1	→ 1	→ 1	→ 3	7	8	→ 8	→ 8	→ 8	→ 8	→ 8	7				
		1, 3, . . .	11			3	→ 4	→ 9	→ 1	→ 1	→ 1	→ 1	→ 1	→ 4	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→ 8	→	8 each
SF/S8	1, 2, 3	1, 3, . . .				1	→ 4						1	→ 4											
		1, 2, 3, . . .	7	→ 8		6	→ 8	→ 6	→ 8	→ 8	→ 8	→ 8	7												
		1, 3, . . .	3	→ 8	→ 8	7		4	→ 8	→ 8	→ 8	→ 8	→ 8	→ 7		8	→ 8	→ 8	→ 8						
		1, . . .				1‡							1‡												

( ) = Weak reaction, with antibody 2 [type 1, (2), 3] or antibody 3 [type 1, 2, (3)]. Usually indicative of mixed culture of different serotypes.
* Each subculture derived from a single colony of the previous one (see Materials and Methods).
† Type 1, 2; but phenotypic, not genotypic: subculture yielded only type 1, 2, 3.
‡ Type 1; but phenotypic, not genotypic: subculture yielded only type 1, 3.

Table 2. *Variations of serotype involving* gain *of antigen* 2 *or antigen* 3

Strain	Serotype of original culture	Serotypes of colonies tested	Serial no. of subculture*... 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 — No. of colonies tested on each subculture (tabulated according to serotype)
41633	1, (2), 3	1, 3, ...	12 → 9
		1, (2), 3, ...	2 → 1 → 9 → 3 → 8 → 8 → 8 → 8 → 8 → 8 → 7 → 8 → 8 → 8 → 8
		1, 2, ...	1 → 4 → 4 → 4 → 4
		1, 2, 3, ...	1 → 8
		1, 2, ...	1 → 4 → 4 → 4 → 4
		1, ...	1 → 8 → 3 → 8 → 7 → 8 → 8 → 8 → 8 → 8 → 8 → 8 → 8 → 7 → 8 → 8
		1, 2, ...	1 → 3 → 4 → 4 → 4 → 4 → 4 → 4 → 4 → 4 → 4
		1, ...	1 → 4 → 4 → 4 → 4
W. 18–323	1	1, 3, ...	1 → 4 → 4 → 4 → 4
		1, ...	3 → 8 → 8 → 8 → 8 → 8 → 8 → 8 → 6 → 8 → 8 → 7 → 8 → 8 → 8 → 8 → 8
		1, 3, ...	1 → 4 → 4 → 4 → 4 → 4
		1, 2, ...	1 → 4 → 4 → 4 → 4

( ) = Weak reaction, with antibody 2 [type 1, (2), 3]. Usually indicative of mixed culture of different serotypes.
* Each subculture derived from a single colony of the previous one (see Materials and Methods).

Another example of phenotypic suppression is seen in strain M21356, in which type 1, 2 growth was found in subculture from a type 1, 2, 3 parent, but reverted to a pure growth of type 1, 2, 3 on the next subculture.

In Table 2, both strains show gain of antigen 2 by type 1. They also show gain of antigen 3, changing 1, 2 to 1, 2, 3 with strain 41633, and changing type 1 to 1, 3 with strain W.18–323. The only gain-mutation that was not detected was the change from 1, 3 to 1, 2, 3.

The relative stability of type 1, 3 strains, indicated by these results, was a feature of other 1, 3 strains studied less extensively than the ones recorded here.

It is also noteworthy that on no occasion did we detect a mutant having antigen 2 and/or 3 but devoid of antigen 1.

DISCUSSION

As we were deliberately looking for evidence of mutation in the serotypes of *Bord. pertussis*, we chose strains from children which were found to be mixed cultures of different serotypes when first tested. This may have been evidence, we felt, that they had already undergone mutation in the child and they may therefore be more fruitful objects of study than the possibly more stable strains which appeared to be pure cultures each of a single serotype. We are not able even to guess at the possible mutation rates of the more stable strains, and we thought that it would be perhaps unnecessary to attempt to determine them, and certainly too laborious and too expensive to do so with the only method available to us.

The mutations involving the loss or gain of antigen 2 or 3 by some of our type 1, 2, 3, type 1, 2, and type 1 strains, were of a high frequency, perhaps similar to the phase variation in the H antigens of salmonellas (Stocker, 1949). Such a mutation rate, of about 10^{-3} or 10^{-4}, would be consistent with our detection of one mutant colony out of eight, after an average of about nine or ten serial single-colony subcultures (Tables 1 and 2). The mutation rate of our type 1, 3 strains was lower than that of the other serotypes: indeed, we did not detect such mutation *in vitro*. But its occurrence *in vivo* is suggested by our studies in marmosets (Stanbridge & Preston, 1974) in which type 1, 2, 3 and type 1 organisms were occasionally isolated some weeks after inoculation of the nasopharynx with a pure culture of type 1, 3.

We also failed to detect a mutant having only antigen 2 or only antigen 3. This was unfortunate, for such mutants would be most useful in the preparation of monospecific pertussis antisera, and in the titration of antibodies 2 and 3 in polyvalent sera. It seems, however, that loss of antigen 1 rarely occurs or is a lethal mutation.

The mutations that we have detected provide an explanation for a number of previously unexpected observations. These include changes of serotype during the course of infection in a child (Preston & Stanbridge, 1972) or marmoset (Stanbridge & Preston, 1974), and the not uncommon isolation of different serotypes in the same household (Public Health Laboratory Service, 1973). They also provide a further explanation, in addition to the difficulties in the technique of typing strains of *Bord. pertussis* (Preston, 1970), for the differing serotype results reported by two or more laboratories on strains isolated in that Public Health Laboratory Service

survey, and for the high proportion of mixed serotypes that were discovered when those strains were re-typed later.

There is a danger of confusion in terminology between 'serotypes' and 'phases'. We would note that our studies refer to the heat-labile agglutinogens of Andersen (1953); and we agree with Cameron (1967) that a parent 'serotype' (1, 2, 3) may pass through intermediates (1, 2 or 1, 3) to the degraded form (type 1). It seems likely that such loss of antigens was responsible for the earlier division of laboratory strains of *Bord. pertussis* into four 'phases' by Leslie & Gardner (1931), freshly isolated 'smooth' strains of phase I degenerating to 'rough' variants of phase IV. However, we have not been able to obtain cultures of phase II, III or IV to test this hypothesis. It seems likely that such cultures no longer exist, and it would perhaps be best that the 'phases' of Leslie & Gardner be forgotten.

Another kind of variation in *Bord. pertussis* was described by Lacey (1960) but was quite distinct from the mutations which we record here. Lacey's 'modes' were phenotypic, the organisms being reversibly changed from X-mode to C-mode by alterations in the conditions of growth. All of our mutants were incubated at a temperature and on a medium that would produce growth in the X-mode.

Lastly, it must be accepted that not all strains of *Bord. pertussis* may be subject to such rapid mutation as we have detected, and that relatively stable strains of each serotype have been maintained in laboratories over many years. Nevertheless, their potential instability has serious consequences. In the preparation of monospecific typing sera, for example, it is essential to test the serotypes of the actual suspensions used for the immunization of animals and those used for the absorption and titration of their sera. Similarly, in the production of pertussis vaccine, it is not sufficient to start with strains having the appropriate range of antigens: the final product must be tested for the presence of all three antigens, in order to guard against both loss-mutation and phenotypic suppression.

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Absorption of hexachlorophane from dusting powder on newborn infants' skin

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SUMMARY

In a maternity hospital in which the umbilicus and trunk of healthy newborn infants were treated with 0·33 % hexachlorophane dusting powder, the hexachlorophane content of blood was measured in mothers before delivery, in infants' umbilical samples at birth, and at 8 days of age in capillary blood samples. One mother and her baby had rather high blood concentrations of hexachlorophane, probably derived from a toilet preparation used before admission to hospital. Hexachlorophane was absent or barely detectable in the other mothers' blood and in the infants' umbilical blood. The hexachlorophane concentrations in the blood of 8-day-old infants ranged from nil to 0·166 μg./ml. (mean 0·066 μg./ml.). These were much less than the concentrations reported to be toxic in animals.

In a previous trial now reported here, a dusting powder containing chlorhexidine instead of hexachlorophane was found to delay the separation of the umbilical cord.

INTRODUCTION

In a preliminary study (Alder, Burman, Corner & Gillespie 1972) small amounts of hexachlorophane were found in blood samples of infants whose skin was treated with 'Ster-Zac' dusting powder (containing 0·33 % hexachlorophane) to prevent staphylococcal infection. The blood concentrations of hexachlorophane were less than those reported to be toxic in animals (Curley *et al.* 1971). It was evident, however, that blood obtained from the umbilical cord and by heel prick were liable to contamination by hexachlorophane on the skin and elsewhere, derived from powder used on infants in the nurseries and on infants and some mothers in the labour room. Hexachlorophane might also reach infants' blood from toilet preparations used by mothers before admission to hospital. Hence it seemed likely that hexachlorophane absorption through the skin was still less than that indicated by the blood analyses, with an even greater margin of safety. This would be consistent with the absence of reported damage to any of the many thousands of infants treated with 'Ster-Zac' powder, in many hospitals, since the method was introduced in Bristol about 18 years ago (Gillespie, Simpson & Tozer, 1958; Baber *et al.* 1967).

We report here a further study of hexachlorophane in infants' blood carried out

in the Bristol Maternity Hospital in 1973, with more stringent precautions to avoid contamination of the samples. Despite the opinion of Plueckhahn (1973) that capillary blood is necessarily unreliable because of contamination, heel-prick samples (also used for Guthrie tests) were again employed. We did not think it justifiable to take venous blood from newborn infants for experimental purposes. The mothers' blood was also analysed to detect hexachlorophane derived from toilet preparations.

The possibility, however remote, that hexachlorophane, even when correctly used, might have toxic effects, makes it relevant to consider other disinfectants as substitutes. One of these, chlorhexidine ('Hibitane') was investigated in Bristol about 13 years ago, but the trial was discontinued because of delay in the separation of the umbilical cords. These results, previously unpublished, are recorded here.

MATERIALS AND METHODS

The routine care of umbilicus and skin was as previously described (Alder *et al.* 1972) except that no hexachlorophane preparations were used by members of staff nor by mothers at any time after admission, and no hexachlorophane was used on infants in the labour room. The umbilical stump was sealed with 'Octaflex' in the labour room but 'Ster-Zac' powder was not applied to the umbilicus and flexures until the infant reached the nursery, within an hour or two of birth.

Three heparinized blood specimens were taken from each mother and her baby: (i) a venous sample from the mother, just before delivery, to detect hexachlorophane derived from toilet preparations used before admission to hospital, (ii) an umbilical cord sample collected in the labour room and (iii) more than 0·5 ml. of capillary blood by heel prick (also used for the Guthrie test), from the infant on the 8th day; before pricking the skin an assistant held the limb free from the bedding while it was thoroughly washed with 70 % alcohol containing 0·4 % ammonia and dried with cotton-wool.

Hexachlorophane in blood was measured as before, by gas/liquid chromatography at the Huntingdon Research Centre (Alder *et al.* 1972). The blank value was between 0·00 and 0·02 μg. per ml. but no adjustment was made for this in the results.

RESULTS

Twenty-two mothers and their babies were investigated. Two babies left hospital before the 8th day, and the capillary blood samples from another three were insufficient. Among those remaining in the investigation, one mother (No. 21) had a high hexachlorophane concentration in her venous sample (0·211 μg per ml.); her baby also had high concentrations in umbilical and heel-prick samples (0·158 μg. per ml. and 0·307 μg. per ml. respectively). These results could not be attributed to the use of hexachlorophane in hospital. The most likely explanation was the presence of hexachlorophane in one of several toilet preparations which this lady admitted to using before admission to hospital. Subsequent enquiry revealed only one that might have been responsible, a 'body mist deodorant'. This preparation was no longer manufactured and was not available for analysis. A blood sample from this

Table 1. *Hexachlorophane (μg./ml.) in mothers' and infants' blood*

		Mothers' venous blood (A)	Infants' blood: Umbilical (B)	Infants' blood: 8th day capillary (C)
Present series (excluding No. 21 – see text)	Mean	0·030 (21)	0·032 (21)	0·066 (16)
	Range	0·000–0·100	0·000–0·104	0·000–0·166
Previous series (Alder *et al.* 1972)	Mean	Not tested	0·044 (15)	0·180 (15)
	Range	Not tested	0·010–0·120	0·040–0·500

(The figures in brackets are the numbers of samples.)

Table 2. *Effect of dusting powders on dates of separation of umbilical cord in Nursery 2, Bristol Maternity Hospital*

	Date (1962)	Powder	No. of babies	Day of separation of cord: 4	5	6	7	8	9	10	11	12 and above
A	8–28 May	'Ster-zac' hexachlorophane	32	0	4	4	8	13	3	0	0	0
B	29 May–10 July	0·2% chlorhexidine	37	0	1	3	5	7	14	5	1	1
C	31 July–31 October	'Ster-zac' base. (No hexachlorophane)	132	6	9	27	42	31	12	4	1	0

Comparisons of separation at 9 days or later.
A and C, $\chi^2 = 0{\cdot}059$, Df = 1. Not significant.
B and C, $\chi^2 = 29{\cdot}455$, Df = 1. Highly significant $P < 0{\cdot}001$.
A and B, $\chi^2 = 14{\cdot}958$, Df = 1. Highly significant $P < 0{\cdot}001$.

mother 16 weeks after delivery contained only 0·02 μg. hexachlorophane per ml., little more than the 'blank' value.

The results for the remaining mothers and babies are summarized in Table 1, with the results of our previous series for comparison. The hexachlorophane concentrations in umbilical blood and maternal blood were little more than the blank value. The values in the 8th day blood samples were slightly higher, but distinctly less than the 8th day values in our previous series. Evidently the precautions to reduce contamination had succeeded and justified the use of capillary blood. It is interesting to note that our results were rather similar to the venous concentrations reported by Plueckhahn (1973) in infants who were washed with hexachlorophane emulsion, although allowance must be made for differences between analytical methods.

Chlorhexidine dusting powder

In 1962 an investigation of dusting powder containing 0·2% chlorhexidine was begun in order to compare its efficiency in preventing staphylococcal sepsis with that of hexachlorophane (K. Simpson, R. C. Tozer and W. A. Gillespie, unpublished). Chlorhexidine powder undoubtedly diminished staphylococcal colonization

of the umbilicus and skin but, unexpectedly, it also caused delay in separation of the umbilical cord stump (Table 2). This effect could perhaps be explained by the wider range of organisms susceptible to chlorhexidine than to hexachlorophane, so preventing bacterial action that may assist the cord to separate. Because the effect sometimes caused inconvenience and delay in patients leaving hospital, the investigation of chlorhexidine powder was discontinued.

DISCUSSION

When investigating the concentration of hexachlorophane in infants' blood it is essential to minimize contamination of the samples by contact with skin or by placental transfer from mothers' blood (Plueckhahn, 1973). The importance of these precautions was well shown by the high concentration of hexachlorophane in the blood of one mother and her baby in our series, probably derived from a toilet preparation used outside hospital; and also by the lower concentrations in infants' blood samples when greater care was taken to avoid contamination than in the previous series.

Very many thousands of normal infants have been treated with 3 % hexachlorophane in emulsion or 0·33 % in dusting powder, in many hospitals, with no evidence of harm. Toxic effects, sometimes fatal, have been associated with topical application of the emulsion to burned or excoriated skin (Pleuckhahn, 1973). Our results and those of Plueckhahn in healthy infants treated in hospital for the first week or so of life with either of these preparations, showed that blood hexachlorophane concentrations were much below the minimum of about 1 μg-per ml. that was found to be toxic when maintained in rats or monkeys for many weeks (Curley & Hawk, 1971; Hart, 1971). Hexachlorophane undoubtedly has prevented much staphylococcal sepsis in infants (Simpson, Tozer & Gillespie, 1960; Gluck & Wood, 1961; Pleuckhahn & Banks, 1963; Baber *et al.* 1967). The incidence of breast abscess in mothers was also reduced (Corner, Crowther & Eades, 1960; Plueckhahn & Banks, 1964). Several authors have recently reported increases in staphylococcal sepsis when hexachlorophane prophylaxis was withdrawn (Ayliffe, Brightwell, Ball & Derrington, 1972; Alexander & Pitkewicz, 1973; Dixon, Kaslow, Mallison & Bennett, 1973). It would be wrong, therefore, to discard hexachlorophane unless an effective and safer substitute is found. One possible substitute, chlorhexidine, had the undesirable effect of delaying separation of the umbilical cord.

Although all evidence shows that hexachlorophane treatment of normal infants' skin is safe when correctly performed it should be realized that hexachlorophane, like other disinfectants, is toxic if misused. Its use therefore should generally be confined to the first 7–10 days of life, in hospital and under medical supervision. Hexachlorophane should not be used on infants with burnt or excoriated skin. Special caution is advised in treating low birth weight babies (Plueckhahn, 1973); but the protection of these babies from staphylococcal sepsis is particularly important and the use of hexachlorophane should be investigated further before deciding upon its safety.

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A controlled trial of inactivated monovalent influenza A vaccines in general practice

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SUMMARY

A trial of influenza A vaccines in general practice is described. Five hundred and seven subjects were vaccinated with either inactivated monovalent A/Hong Kong vaccine, A/England vaccine or influenza B vaccine as control. Local reactions were noted in 24% and general reactions in 12% of patients. Antibody titres in serum were measured by haemagglutination inhibition (HI) and complement fixation (CF) tests in 465 subjects. The influenza vaccines produced substantial increases in both homologous and heterologous antibodies as measured by the HI test and a comparatively poor response as measured by the CF test. Although clinical influenza was confirmed in only a few cases, there was serological evidence of significant subclinical infection in the control group.

INTRODUCTION

Despite widespread use there is still disagreement about the place of influenza vaccine in the control of influenza. The majority of vaccine trials have involved defined populations such as Service personnel, industrial workers, university students and pupils in boarding schools. Few studies have been reported from family practice. This paper presents such a study. With the collaboration of members of the Royal College of General Practitioners a trial was arranged to assess the efficacy of inactivated monovalent influenza A vaccines against expected influenza in the winter of 1972/73. The investigation included an adequate control group inoculated with influenza B vaccine, and laboratory facilities for the virological investigation of suspected cases and the measurement of antibody response to the different vaccines. In the event there was little influenza in Leicester during the winter and it was not possible to assess the protective efficacy of the vaccines. However, the vaccination reactions, antibody response and virus isolations observed in the trial are described below.

MATERIAL AND METHODS

The study was carried out in patients from 25 general practices in Leicestershire who had agreed to participate and to give the necessary blood samples. A first sample was withdrawn towards the end of November 1972 when patients were given one of three monovalent vaccines by random allocation. A second sample was withdrawn three weeks later and a final sample at the end of the following March. Participants were asked to report any symptoms of influenza and those who did so had a throat swab taken for virological examination. Acute and convalescent blood samples were also requested.

Vaccines

The three inactivated monovalent vaccines which were prepared by Evans Medical Limited and supplied by the Epidemiological Research Laboratory, Colindale, were as follows:

1. Vaccine A/HK contained A/Hong Kong/1/68(H3N2) (recombinant X31).
2. Vaccine A/Eng contained A/England/42/72(H3N2) (recombinant XPR8).
3. Vaccine B contained B/Victoria/98926/70.

Administration

The identity of each vaccine was concealed by a code number known only to the laboratory. Each vaccine was given intramuscularly, the A vaccines in a dose of 600 i.u. and the B vaccine in a dose of 400 i.u. The vaccines were allocated according to day of birth; those born on the 1st–10th day of any month received A/HK, on the 11th–20th B, and on the 21st–31st A/Eng vaccine.

Reactions to vaccination

On returning about three weeks later for the post-vaccination sample the patient was asked about local and general reactions. These were recorded as nil, moderate or severe. Locally a moderate reaction was characterized by slight swelling and aching at the site of injection. A reaction was recorded as severe when the arm became painful, red and swollen. Constitutionally a moderate response was characterized by slight pyrexia and mild aching of the back and limbs and a severe reaction by influenza-like symptoms. In the event of illness the date and details of symptoms were recorded by the general practitioner.

Serological methods

HI tests were carried out in microplates using 0·025 ml volumes of serum and equal volumes of virus containing eight haemagglutinating units. Each serum was tested for HI antibodies against A/Hong Kong/1/68(H3N2), A/England/42/72(H3N2) and B/England/21/68 viruses.

CF tests were carried out according to the method described by Bradstreet & Taylor (1962) modified by the use of a microtitre technique.

Table 1. *Age and sex distribution of vaccinated patients*

Sex	Age < 25 years	25–34 years	35–44 years	45–54 years	55–64 years	⩾ 65 years	Total
Male	15	35	50	43	27	16	186 (40 %)
Female	23	63	72	58	35	28	279 (60 %)
Total	38	98	122	101	62	44	465

Virus isolation

Throat swabs in transport medium were inoculated within 24 hr of collection in rhesus monkey kidney tissue culture cells supplied by the Biological Standards Division of the National Institute of Medical Research. The cells were maintained in mixture '199' without serum. The monkey kidney cells were tested for haemadsorption after 2, 4, 7 and 14 days, and examined for the presence of cytopathic effect. Each specimen was also inoculated in the Bristol line of HeLa cells and human embryo lung cells for the isolation of other respiratory viruses.

RESULTS

A total of 507 patients from the 25 practices entered the trial. Any patient who failed to supply at least two blood samples was excluded and consequently 465 patients qualified for inclusion in the analysis.

All but six of the patients were adults. The age and sex distribution is shown in Table 1. There were more females than males, but the age distribution was similar for both.

One hundred and forty one (30 %) patients were given A/HK vaccine, 155 (33 %) B vaccine and 169 (36 %) A/Eng vaccine. The slight excess in the A/Eng group was expected because of the extra days of birth allocated to this group. The method of allocation appears to have been successful since the three groups were of comparable size with similar age and sex composition and also similar pre-vaccination antibody titres.

Reactions to vaccination

Information on local reactions was recorded for all but 20 of the 465 patients. Ninety-seven (22 %) reported mild, and 12 (3 %) severe local reactions, which were slightly more frequent in the younger age groups. Information on general reactions was recorded for all but 15 of the patients. Forty-two (9 %) reported mild and 11 (2 %) severe general reactions. The rates were similar in all age groups. Each of the three vaccines gave similar reaction rates.

HI antibody results

Pre-vaccination results

Many patients had HI antibody to A/HK at the time of vaccination (Table 2). Of 463 patients 306 (66 %) had antibody titres of 20 or more. In contrast only 137 (30 %) had antibody to A/Eng. In 264 patients the A/Eng antibody titre was

Table 2. *Pre-vaccination HI antibody titres to A/HK and A/Eng*

			A/HK antibody Number of patients with titre					
			⩽ 10	20	40	80	⩾ 160	Total
A/Eng antibody	Number of patients with titre	⩽ 10	153	77	54	28	14	326
		20	3	6	27	18	10	64
		40	1	2	8	13	8	32
		80	—	—	1	3	15	19
		⩾ 160	—	1	1	1	19	22
		Total	157	86	91	63	66	463

Table 3. *Changes in HI antibody titre to A/HK and A/Eng in* 137 *patients after inoculation with A/HK vaccine*

	HI antibody to A/HK		HI antibody to A/Eng	
Antibody titre	Before vaccination	After vaccination	Before vaccination	After vaccination
⩽ 10	52	5	101	20
20	25	3	13	13
40	21	7	10	22
80	21	29	6	25
160	8	21	2	28
320	3	29	3	10
640	4	21	1	14
⩾ 1280	3	22	1	5
Total patients	137	137	137	137
Geometric mean titre	38	310	15	110

lower than their A/HK titre, in 189 patients it was the same and in only 10 was it higher. Despite this tendency for antibody titres to A/Eng to be lower there was a correlation with the A/HK antibody since patients with high A/HK titres were more likely to have high A/Eng titres than those with low A/HK antibody.

The number of patients with HI antibody to influenza B was smaller, only 23 % having titres of 20 or more.

An analysis not presented in the table showed that the proportion of patients with A/HK antibody was similar in all age groups, whereas the proportion of patients with antibody to A/Eng increased slightly with age – in the ⩾ 65 age group 34 (45 %) had antibody to A/Eng, a proportion which was significantly greater than the 30 % at all ages ($P < 0{\cdot}02$). Antibody to influenza B was proportionally greater (41 %) in the group under 25 years old ($P < 0{\cdot}001$).

Post-vaccination results

Of the 141 patients who received A/HK vaccine, 137 had pre- and post-vaccination samples tested. All but 5 of the 52 patients without A/HK antibodies before vaccination (i.e. titres of 10 or less) developed A/HK antibody after vaccination (table 3). In most patients with pre-vaccination antibody the titre rose substantially. The average increase in titre was about 8-fold; the geometric mean titre

Table 4. *Changes in HI antibody titre to A/HK and A/Eng in 164 patients after inoculation with A/Eng vaccine*

Antibody titre	HI antibody to A/HK		HI antibody to A/Eng	
	Before vaccination	After vaccination	Before vaccination	After vaccination
⩽ 10	51	11	111	5
20	33	9	24	12
40	32	8	13	15
80	16	13	6	18
160	16	22	8	32
320	9	27	1	22
640	5	31	—	23
⩾ 1280	2	43	1	37
Total patients	164	164	164	164
Geometric mean titre	43	370	16	310

Table 5. *Increase in influenza A antibody titres after vaccination with A/HK and A/Eng vaccines in patients with and without pre-vaccination antibody*

Presence (+) or absence (−) of pre-vaccination HI antibody to (pre-vaccination GMT)		Number vaccinated	Vaccine given	Increase in titre to	
				A/HK	A/Eng
A/HK− (GMT < 10	A/Eng− GMT < 10)	51	A/HK	× 16	× 8
A/HK+ (GMT 56	A/Eng− GMT < 10)	50		× 8	× 16
A/HK+ (GMT 172	A/Eng+ GMT 81)	35		× 2	× 2
A/HK− (GMT < 10	A/Eng− GMT < 10)	51	A/Eng	× 12	× 16
A/HK+ (GMT 60	A/Eng− GMT < 10)	60		× 12	× 64
A/HK+ (GMT 145	A/Eng+ GMT 60)	53		× 6	× 6

(GMT) rose from 38 to 310. Vaccination with A/HK also induced the development of A/Eng antibody (Table 3). Thus among the 137 patients 101 had no pre-vaccination antibody to A/Eng and of these 81 developed antibody. Again there was an average 8-fold increase; the GMT rose from 15 to 110.

Of the 169 patients who received A/Eng vaccine 164 had pre- and post-vaccination samples tested. All but five of the 111 patients without A/Eng antibody before vaccination developed antibody after vaccination, and in most patients there was a substantial increase in titre (Table 4). The average change was nearly 20-fold; the GMT rose from 16 to 310. Vaccination with A/Eng was also associated with the development of A/HK antibody (Table 4). Thus among these 164 patients there were 51 without pre-vaccination antibody to A/HK and all but 11 of these developed antibody after vaccination. There was an average increase of just over 8-fold; the GMT rose from 43 to 370.

Table 6. *Changes in HI antibody to B in 151 patients after inoculation with B vaccine*

Antibody titre	HI antibody to B	
	Before vaccination	After vaccination
⩽ 10	114	35
20	22	22
40	9	29
80	5	25
160	1	20
320	—	9
⩾ 640	—	11
Total patients	151	151
Geometric mean titre	13	65

The antibody response to each vaccine was affected by the presence or absence of antibody to A/HK and A/Eng before vaccination. The responses are summarized in Table 5 which shows the post-vaccination changes in titre according to the status of pre-vaccination antibody. It is clear that each vaccine produced substantial increases in both homologous and heterologous antibodies. The increases were less in the groups of patients who already had antibody to both antigens and whose pre-vaccination GMT of A/HK antibody was the highest. However, the A/Eng vaccine tended to be more effective in producing antibody to the A/Eng strain.

It is of interest that the presence of antibody to A/HK in the absence of antibody to A/Eng before vaccination was associated with a significantly better response to A/Eng antibody. This feature was seen after the administration of each vaccine.

Response to the vaccine containing the B strain was less than to vaccines containing the A strains. Of the 155 patients allocated B vaccine 151 had pre- and post-vaccination samples tested (Table 6). There were 114 patients without pre-vaccination antibody and 35 still had no antibody after vaccination. The average change was just over 4-fold; the GMT rose from 13 to 65.

End-of-trial results

Antibody titres tended to fall slightly during the three months or so between the post-vaccination and final samples. Fig. 1 shows average antibody titres to A/Eng for each of the three vaccination groups at the three stages of the trial. A slight increase in average titre among patients receiving B vaccine suggests that some of these patients were infected with A/Eng virus around the time of the start of the trial; of the 155 patients in the B vaccination group 15 showed pre- to post-vaccination 4-fold rises in titre to A/Eng, and three more patients showed similar rises during the post-vaccination to end-of-trial period.

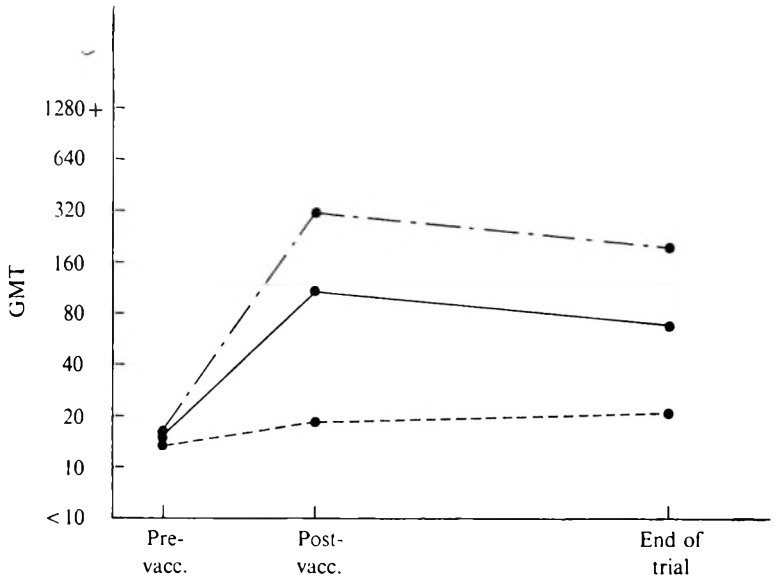

Fig. 1. Average HI antibody titres to A/England at each stage of the trial, according to vaccine given. Vaccine given: ———, A/HK; — - —, A/Eng; - - - - - -, B.

CF antibody results

Many patients had CF antibody to influenza virus at the time of vaccination. Of the 463 entry specimens tested 302 (65 %) had titres of 8 or more to A antigen and 245 (53 %) to B antigen.

Of the 141 patients given A/HK vaccine 137 had pre- and post-vaccination tests for CF antibody. These included 36 with no antibody to A antigen before vaccination (i.e. titres of 4 or less) and 25 of these still had no antibody three weeks after vaccination. Similarly with the 169 patients given A/Eng vaccine 158 had both samples tested; 55 had no pre-vaccination antibody and 44 of these did not acquire antibody after vaccination. Among all patients receiving an A vaccine the average increase in titre was barely 2-fold; the GMT rose only from 21 to 34.

One hundred and fifty one patients given B vaccine had pre- and post-vaccination samples tested by CF. There were 63 patients without pre-vaccination antibody to B antigen and 25 of these still had no antibody after vaccination. The average change in antibody was about 2-fold ; the GMT rose from 11 to 20.

Only slight falls in average of CF antibody were seen over the post-vaccination to end-of-trial period. Fig. 2 shows average A antibodies at the three stages of the trial in each of the three vaccine groups. The group receiving B vaccine, which acts as a control group, showed virtually no change over the whole period.

Comparison of HI and CF antibodies

Table 7 shows responses as measured by the two methods in patients receiving either of the A vaccines. Fewer patients showed response to vaccination by the CF tests as compared with HI tests. Of 310 patients whose paired sera were tested by both methods, only 47 (15 %) showed a 4-fold or greater rise in titre by the CF test as compared with 235 (76 %) by the HI test.

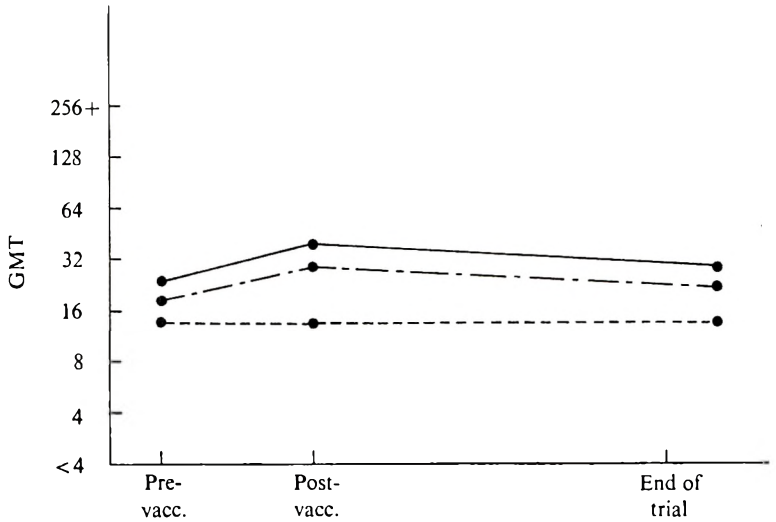

Fig. 2. Average CF antibody titres to influenza A at each stage of the trial, according to vaccine given. Vaccine given: ———, A/HK; — - —, A/Eng; - - - - - -, B.

Table 7. *Increase in antibody titre in* 301 *patients against influenza A virus as measured by CF and HI tests*

Increase in titre	CF test	HI test
No increase	174	31
× 2	80	35
× 4	27	41
× ⩾ 8	20	194

Patients reporting 'influenza-like' illnesses

Thirty-two (7 %) of the 465 patients reported an influenza-like illness during the period of the trial. Only four cases were confirmed by laboratory examination to be A/Eng infections; three gave positive isolations from throat swabs and the fourth had an 8-fold rise in CF antibody. Two of these cases had received B vaccine, one A/HK vaccine and one A/Eng vaccine. However, in this last patient symptoms had already begun when the vaccine was given. All four cases were reported in November and December.

The remaining 28 cases occurred at intervals throughout the period of the trial. Nine of these had received A/HK vaccine, 11 A/Eng vaccine and 8 B vaccine. These patients were representative of all patients in the trial in respect of age and sex distribution and pre-vaccination titres. Only four of these 28 cases yielded virus from the throat swab. The viruses were identified as rhinovirus (2), respiratory syncytial virus (1) and herpes simplex virus (1). Serological examination of acute and convalescent sera has so far been confined to influenza antigens.

Symptoms were recorded and some differences were observed between the probable influenza cases and the rest, but the numbers are very small and not statistically significant. All four influenza cases reported aching limbs, headache and cough and three reported fever and rigors.

DISCUSSION

Influenza vaccines have frequently been assessed by field trial but there is still disagreement about their place in influenza control. Usually influenza vaccine trials are made in factory populations or in other defined communities. The present study, however, was made in family practice. It incorporated the features essential for an adequate assessment of influenza vaccine – a control group chosen by an effective random method and facilities for laboratory examination of cases of influenza-like illness. Laboratory facilities were also available to examine the antibody response to the vaccines used.

In the present study there was a wide age distribution in volunteer patients; the greater proportion of females appears to be similar to that usually found in surgery consultations. Of this population a large proportion must have been infected with A/HK influenza since two-thirds had HI antibody to this strain. A considerable, though smaller proportion had also been infected with the A/Eng strain. The proportion with this antibody – 30 % – was similar to that found by Pereira *et al.* (1972) and presumably reflects the presence of the virus in England during the previous months. However, it is difficult to see how recent infection could account for the greater proportion of older people with antibody to the virus. This excess in the older patients was considerable and attained statistical significance. This finding suggests that the older group may have been infected with a strain antigenically similar to the A/Eng strain earlier in life. If this is so such strains have presumably circulated in England in the past, before identification of influenza strains became possible.

In contrast to the age distribution of A/Eng antibody, HI antibody to the 1968 influenza B strain was more common in persons under 25. Influenza B tends to be seen most commonly in the young, and so the older group in this trial had presumably escaped infection with this strain.

Like most inactivated influenza vaccines, the two monovalent A vaccines produced a good HI antibody response to the homologous virus strain. Each vaccine, however, also produced a good response to the heterologous strain. Both virus strains clearly include some closely similar antigens. The findings show that the A/Eng antibody response with either vaccine is best in persons who already possess antibody to the A/HK strain. This response was also seen in the studies by Pereira *et al.* (1972) and Hoskins *et al.* (1973).

The large proportion of entrants with CF antibody against influenza A (65 %) is surprising even despite the occurrence of influenza A virus in the community just before and at the start of the trial. According to Hoyle & Fairbrother (1947) a CF antibody titre of 16 or higher is practically diagnostic of recent infection, while Fairbrother & Martin (1938) claim that such titres decline rapidly within six months. In the present study 266 (57 %) had a pre-vaccination titre of 16 and higher, and four months after vaccination there was little decline in titre. These findings suggest that the mere presence of CF antibody even at a titre thought to be significant cannot necessarily be taken as reliable evidence of a recent influenza infection.

The slight rise in CF antibody titres observed in those patients who received

the A vaccines (Fig. 2) was probably a response to vaccination rather than to infection since there was no comparable rise in CF antibody titre against influenza A in the control group.

The comparatively poor development of CF antibody with inactivated vaccine confirms the observation made by Hoyle & Fairbrother (1947) that the complement fixation test is not suitable to assess the antibody response to vaccination with influenza virus.

The routine influenza A virus reports from the Leicester Public Health Laboratory during the winter of 1972/73 were few in number and occurred almost entirely during November and December when the trial was starting. There was very little influenza diagnosed routinely during the follow-up period of the trial. The infrequency of clinical influenza was substantiated by the results of the trial in which only four of the 32 patients with influenza-like symptoms were shown to have been infected with influenza A virus. Of the remaining 28 patients, four yielded rhinovirus, respiratory syncytial virus and herpes simplex virus; some may have been due to influenza since their illnesses occurred in December and they showed an increase in antibody during the convalescent period, but this increase may also have been the result of vaccination. These findings underline the necessity for laboratory examination of suspected cases in trials of influenza vaccine. Had the present study been dependent on 'clinical' influenza the findings would have suggested that the vaccines did not protect, a conclusion which would have been unwarranted in view of the very few cases of proved influenza which occurred. Indeed a recent study (Hoskins *et al.* 1973) has clearly shown the efficacy of influenza vaccine containing the A/HK strain in protecting against A/Eng influenza.

It is of interest to note that in the control group 16 (10%) of the patients showed serological evidence of subclinical infection with influenza A virus as compared with only two overt cases. It would appear that at a time when influenza was apparently limited in Leicester the number of subclinical infections was proportionately much greater than the number of known cases. Miller *et al.* (1973) reported a survey in which about half of those persons with serological evidence of influenza A/HK infection during 1968/70 reported no illness. Our findings would indicate that subclinical infection is probably more prevalent than is generally recognized.

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