Vol. 73, No. 1 August 1974

THE JOURNAL OF HYGIENE

(Founded in 1901 by G. H. F. Nuttall, F.R.S.)

CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London NW1 2DB

American Branch: 32 East 57th Street, New York, N.Y.10022

1974 *subscription* £16.00 *net* (*US* $52.00) (6 parts)

Single parts £3.50 *net* (*US* $11.50)

The Journal of Hygiene

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Single parts £3.50 net (US $11.50 in the U.S.A. and Canada) plus postage.

Three parts form a volume; two volumes are published per year. The 1974 subscription price of a volume (which includes postage) is £8.00 net (US $26.00 in the U.S.A. and Canada).

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J. Hyg., Camb. (1974), **73**, 1
Printed in Great Britain

Air sampling of smallpox virus

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(*Received* 30 *August* 1973)

SUMMARY

Airborne smallpox virus has been recovered in an isolation hospital using an adhesive surface sampling technique in the presence of very low aerosol concentrations. Previous work in this field is reviewed. Successful recovery of airborne virus depends on sampling large volumes of air with a suitable sampler and thorough investigation of the whole sample taken for the presence of viable virus. More information on the characteristics and behaviour of airborne smallpox virus is needed in particular with regard to the future design and siting of smallpox isolation units.

INTRODUCTION

It is generally accepted that a patient with smallpox becomes infectious when the first external signs of disease appear. Virus is usually plentiful in the respiratory and oral secretions (Downie *et al.* 1961) and also in skin lesions. Smallpox is most frequently transmitted in the early stages of the disease during close contact but infection may also occur through handling infected clothing and bedding (Cramb, 1951; Dixon, 1962). Outbreaks of smallpox have occurred when contact with known infected individuals or families could not be established (Power, 1882; Barry, 1889; Peirce, Melville, Downie & Duckworth, 1958; Perkins & Vaughan, 1961; Dixon, 1962; B.M.J., 1970). There is evidence which suggests that dissemination of infection to remote parts of a hospital may have occurred by airborne transmission (WHO, 1970). Accidental laboratory infection with smallpox is rare but a recent incident, which gave rise to two fatal secondary cases, appears to have occurred through exposure to an infective aerosol.

Immunization of the general population is no longer encouraged, largely because of the incidence of serious side effects, but the introduction of smallpox through air travel is an ever present possibility despite world-wide attempts to eradicate the disease (Henderson, 1973).

The study of the behaviour of airborne smallpox virus has not received much attention and there are few references in the literature (Meiklejohn *et al.* 1961; Downie *et al.* 1965). Despite the plentiful presence of smallpox virus in oral secretions, skin lesions and contaminated bedding the reported air sampling results appeared to indicate low air concentrations of virus. Rabbitpox virus has been successfully sampled by an adhesive surface sampling technique (Thomas, 1970*a*, *b*)

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and it is the application of this method to the sampling of airborne smallpox virus arising from a human source which is reported in this paper.

MATERIALS AND METHODS

Samplers

Slit sampler (Casella) using plates coated with S.G.B. mixture (sucrose, glycerol, and bovine serum albumin; Thomas, 1970*a*), Porton impingers (May & Harper, 1957) containing 10 ml. of '199' medium with antifoam and sedimentation plates coated with S.G.B. mixture were used.

Virus culture

A HeLa cell line (Appleyard & Westwood, 1964) was used for preliminary culture of virus recovered in S.G.B. plates (Thomas, 1970*a*). The cells were harvested after 7 days, disrupted by freezing and thawing and then inoculated in 0·4 ml. amounts into 11-day-old embryonated hen's eggs (5 eggs for each plate). Pirsch & Purlsan (1962) have described hyperplastic foci in HeLa cell monolayers caused by variola virus. The monolayers in the experiments described here were examined before harvesting for evidence of cytopathic effects but none of the changes seen were considered sufficiently well characterized to indicate variola virus. In addition air sampling collected particles of many types some of which may have caused changes in the HeLa cell monolayers which were in no way related to variola virus replication. Impinger samples were inoculated in 0·2 ml. volumes into hen's eggs. All the eggs were incubated for 5 days before being examined for the presence of pocks. Any membranes which showed any changes suggestive of pock formation after incubation were passaged a second time.

Air sampling procedures

Air sampling was carried out for 2 days in two wards (male and female) of a smallpox isolation hospital at the end of a variola minor outbreak. The female ward contained three adult patients only one of whom still had a few dry lesions ('seeds') which were slow in separating. In the male ward were two children awaiting discharge and an adult male who still had a number of active lesions which were considered to be secondarily infected. Air samplers were used within a few feet of the occupied beds in both wards but the sedimentation plates were exposed in groups approximately 20 ft. away from these beds. Sampling periods of $\frac{1}{2}$ to 1 hr. per plate were used with the slit sampler operating at a flow rate of 1 ft.^{3}/min. while the sedimentation plates were exposed for many hours at a time. The impinger samplers were operated at a flow rate of 11·5 l./min. for 15 min. per sample. A summary of the sampling procedures is given in Table 1.

Table 1. *Summary of air sampling procedures undertaken in the male and female wards of the smallpox isolation hospital*

Sampler	Ward	Day	No. of samples	Period of sampling (hr.)	Volume of air sampled	
					ft.³	l.
Slit sampler	Male	1	9	8·25	495	14,008·5
		2	14	7·5	435	12,310·5
Slit sampler	Female	1	10	9·25	555	15,706·5
		2	2	1	60	1,698
Impingers	Male	2	6	1·5	36·6	1,035
Impingers	Female	2	4	1	24·4	690
Sedimentation plates	Male	1, 2	11	36	—	—
Sedimentation plates	Female	1, 2	7	39·25	—	—

Table 2. *Detection of variola virus in the samples taken with the slit sampler in the male ward*

Day	Sample no.	No. of eggs (5 eggs/sample) showing variola pocks	
		First passage	Second passage
1	1	1	0
	2	2	5, 5
	3	3	5, 5
	4	0	—
	5	1	0
	6	0	—
	7	0	—
	8	3	5, 5, 5
	9	4	5, 5, 5, 5
2	1	0	—
	2	1	0
	3	0	—
	4	0	—
	5	2	5, 5
	6	2	5, 5
	7	0	—
	8	2	5, 5
	9	2	5, 5
	10	0	—
	11	0	—
	12	3	0, 0, 0
	13	3	5, 5, 5
	14	2	5, 5

Table 3. *Detection of variola virus in the samples taken with the slit sampler in the female ward*

Day	Sample no.	No. of eggs (5 eggs/sample) showing variola pocks: First passage	Second passage
1	1	2	0, 0
	2	0	—
	3	3	0, 0, 0
	4	2	0, 0
	5	2	0, 0
	6	2	0, 0
2	1	0	—
	2	1	0
	3	1	0
	4	1	0
	5	0	—
	6	0	—

Table 4. *Detection of variola virus in sedimentation plate samples taken in the male ward*

Period exposed	Sample no.	No. of eggs (5 eggs/sample) showing variola pocks: First passage	Second passage
11.45 to 19.45	1	2	0, 0
	2	1	0
19.00 to 07.00	3	4	5, 5, 5, 5
	4	2	0, 0
	5	3	5, 5, 5
12.00 to 20.00	6	2	5, 5
	7	0	—
	8	1	0
	9	0	—
	10	2	0, 0
	11	0	—
	12	1	0

RESULTS

Slit sampler

Pock formation characteristic of smallpox virus was seen only in samples taken in the male ward where 10 of the original air samples taken with the slit sampler were shown to have contained smallpox virus. None of the samples in the female ward were positive. The results of these investigations are given in Tables 2 and 3.

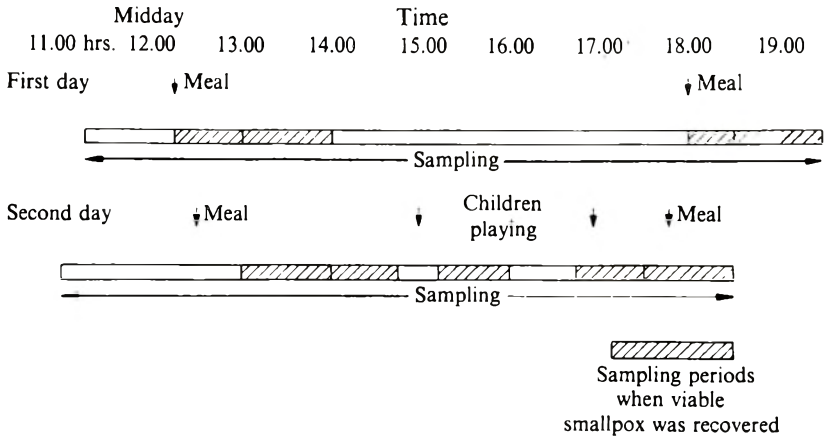

Fig. 1. The diagram indicates the periods when smallpox virus was detected in the air samples taken with the slit sampler in the male ward.

Sedimentation plates

Eleven plates were exposed in the male ward and seven plates in the female ward during the two days of sampling. Again only the male ward samples were positive and the results are given in Table 4.

Impinger samplers

Six samples were taken in the male ward and four in the female ward. No evidence of smallpox was found in any of these samples.

DISCUSSION

Airborne variola virus was detected in the slit sampler plates and in the sedimentation plates only in the male ward. At the time of sampling the adult male patient alone was considered to be infectious since he still had a large number of skin lesions which were separating slowly. Many appeared to be secondarily infected with consequent breakdown of the lesions. On both days successful recovery of airborne virus with the slit sampler occurred during very similar periods which coincided with the main time of activity in the wards. It is the movements of the adult male patient which can best be related to the air sampling results. He did not rise for breakfast but did so just before lunch, about 12.20 p.m. when his bed was made, and similarly in the late afternoon for the next meal. On both days it can be seen in Fig. 1 that airborne virus was recovered during these two similar periods.

The two male child patients were rather subdued on the first day but on the second day in the afternoon they indulged in a wild romp which involved leaping on and off the bed of the adult male. It is possible that this additional activity gave rise to the positive samples obtained in the middle of the afternoon of the second day (Fig. 1).

The results of air sampling with the slit sampler in the male ward given in

Table 2 are based on unequivocal variola pock formation in the second egg passage. It is not possible to place any reliable quantitative interpretation on these results, only the presence or absence of viable airborne virus.

Table 4 shows that there were two positive sedimentation plates out of the three exposed overnight in the male ward and one positive sample was obtained during the daytime exposures. The results are too few to place any useful interpretation on the findings, other than that viable airborne virus was carried well down the ward. The plates were deliberately placed some distance away from the possible source of virus since fall-out of infected particles at close range would be certain to occur as has already been observed by other workers (Downie *et al.* 1965). The exposure of many more plates would have been helpful but the demands of continuous air sampling limited the numbers of plates available for use in this way.

The impinger samples were all negative. One was taken during the period when successful recovery occurred with the slit sampler. Because of the very small quantity of airborne virus it is probable that the 15 min. period of sampling for the individual impingers was too brief. In addition, only a tenth of the total volume of the sampling fluid was tested. Westwood, Boulter, Bowen & Maber (1966), sampling for rabbitpox with impingers also failed to recover any viable virus. Downie *et al.* (1965) were successful in collecting airborne smallpox using impingers. The number of patients and consequently the concentration of airborne virus however was considerably greater than that present in the isolation hospital where the air sampling described in this paper was carried out.

Meiklejohn *et al.* (1961) carried out sampling for airborne smallpox virus with small glass funnels containing tightly packed dry cotton wool. Thirty-eight samples were taken, many in close proximity to acutely ill patients. During several sampling periods agitation of bed clothes and vigorous sweeping of floors was carried out to create an aerosol. Only one of the samples proved positive, despite the large volumes of air sampled (4500–9000 l. of air/sample). This method of sampling is not very efficient for collecting small particle sizes in the respirable range, i.e. those less than 10 μm. in size. The attempts that were made to collect particles (droplet nuclei) expelled from the mouths of patients with oral lesions would not be likely to be successful (W.H.O., 1964) while the likelihood of survival of virus in any such particles collected on the cotton wool would be diminished by the drying effect (Lovelock, Porterfield, Roden & Sommerville, 1952).

Downie *et al.* (1965) reported air sampling for smallpox virus under conditions very similar to those described by Meiklejohn *et al.* Porton impingers, sedimentation (settle) plates and the top stage of an Andersen sampler (Andersen, 1958) were used. Virus was recovered with the first two types of sampler but none with the third which was functioning in this case as a simple sieve sampler with a very low collection efficiency. The presence of virus in the air samples was investigated by inoculating small volumes into embryonated hen's eggs without preliminary concentration. Of the impinger samples 11 %, and of the sedimentation plate samples 40 % were positive. Large droplets expelled from patients' mouths would fall out quickly into the sedimentation plates but only a proportion of the much

smaller airborne droplets would be collected by the impinger. Tyler & Shipe (1959) found the impinger as efficient as other samplers for fine relatively monodisperse aerosols but for heterogeneous airborne particles, such as those in natural aerosols, other types of sampler were more efficient.

Downie and his colleagues also examined the levels of airborne virus arising from bedding contaminated by skin lesions and in this part of the investigation 55% of the air samples were positive, mainly the sedimentation plates. Bed clothes contaminated with discharges from skin lesions provide a much larger source for the dispersal of virus.

The results for air sampling given in the papers by Meiklejohn *et al.* (1961) and Downie *et al.* (1965) reviewed above appear to indicate that despite the very plentiful presence of smallpox virus in oral secretions, skin lesions and contaminated bedding, concentrations of airborne virus close to the patients were not particularly high and that most of the particles carrying smallpox virus tended to sediment rapidly. The slit sampler used in the author's investigations has a high collection efficiency for particles of 1 μm. and above, superior to that of the cotton wool filter used by Meiklejohn *et al.*, and more suitable for sampling heterogeneous natural aerosols than the impingers used by Downie *et al.* The adhesive surface sampling technique has the added advantage of obtaining preliminary replication in HeLa cells before egg inoculation. In this method all the particles carrying viable virus collected in the sample are brought into contact with susceptible tissue cells without further need for manipulation. The method was assessed before use by examining dilutions (up to 10^{-8}) of suspensions of smallpox virus. Virus was not detectable by direct inoculations into eggs at 10^{-6}, 10^{-7} and 10^{-8} dilutions but was readily demonstrated in eggs after preliminary passage of these dilutions in HeLa cells. The combination of an efficient sampler and a sensitive detection method enabled the recovery of viable smallpox virus from very low airborne concentrations.

Hospitals for the isolation of smallpox patients in the past were usually sited in the country away from centres of population. Many of these hospitals are now very old and are in need of replacement. There has been a move in recent years to site new isolation units adjacent to general hospitals, sometimes in areas of high population. These units require extensive and complex air filtration systems to ensure containment of airborne infectious material. The author has undertaken commissioning trials of one such isolation unit. The results of tests on the air filtration system, coupled with the slowly growing body of knowledge concerning the characteristics and behaviour of airborne smallpox virus, clearly indicated a need for very careful siting, construction, and maintenance of the units together with periodic tests of the integrity and efficiency of the air filtration systems.

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J. Hyg., Camb. (1974), **73**, 9
Printed in Great Britain

The incidence of *Brucella* infections in producer-retailer herds in North Lancashire from 1965 to 1972

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(*Received* 17 *October* 1973)

SUMMARY

The results are presented of testing untreated producer–retailer herd milk samples for the presence of *Brucella abortus* during the period 1965–1972 in the North Lancashire region.

There was a steady decline in the incidence of infected herds from 22% in 1965 to 12% in 1971. A sharp fall to 5% in 1972 suggests that the introduction of the Brucellosis Incentives Scheme and the eradication programme has helped to reduce the practice of selling brucella-infected cattle in the open market which was prevalent in the period 1965 to 1970.

This practice of selling brucella-infected cattle may also be a prime factor in the changing pattern of distribution of the biotypes of *B. abortus* which was observed during the period 1965 to 1970.

A comparison of the two areas in the region show that the incidence of herd infection was always greater in the area containing flying herds than in the area in which self-contained herds predominated.

INTRODUCTION

In the United Kingdom brucellosis is primarily a disease of cattle and infections in other animals and man are invariably associated with infected cattle or their products, except for the occasional infection which is acquired either abroad or in the laboratory. An important vehicle in the transmission of the disease from cattle to man is untreated, brucella-infected milk. Whilst the pasteurization of milk is a reliable method for protecting the consumer, the eradication of brucella infection in cattle is the only measure which will eliminate human infections contracted by direct contact with infected animals.

In Lancashire there are approximately 1000 producer-retailer herds, the majority of which are in the north of the county supplying untreated milk directly to the public. In this report an account is given of the incidence of brucella infection in producer–retailer herds in the area served by this laboratory (North Lancashire) since 1965.

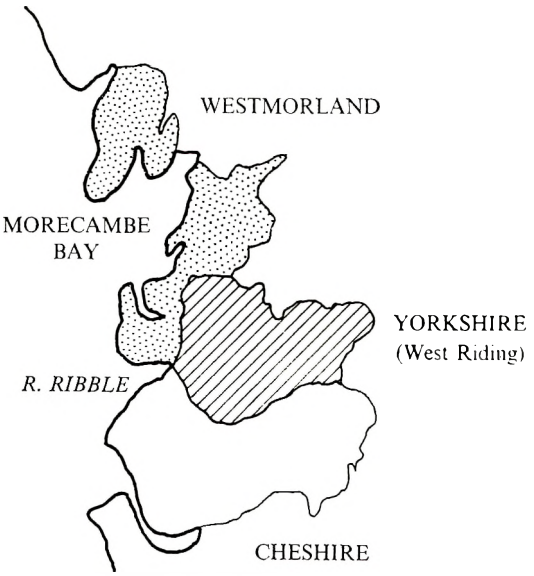

Fig. 1. Lancashire showing Area 1 ▦ mainly self-contained herds and Area II ▨ mainly flying-herds.

MATERIALS AND METHODS

The region under investigation can be divided into two areas designated I and II, as shown in Fig. 1. In Area I most of the producer–retailer herds are self contained units and replacements are reared from their own stock in contrast to Area II where the majority are 'flying herds', replacements being bought as required. Samples of bulk milk from these herds were screened with the milk ring test (MRT) (Report, 1956) and MRT-positive samples were cultured on selective media, and inoculated into guinea-pigs.

Direct culture

The milk samples were transferred to sterile, stoppered test tubes (7 × 1 in). Each sample was kept overnight in the refrigerator (approximately 4° C.) and a sample of gravity cream, withdrawn with a spiral wire (Mair, 1955), was spread over the selective agar with a bent, sterile, glass rod. During the period of the investigation a number of selective media were used and included media described by Mair, 1955; a modification of the medium described by Morris, 1956; Ryan, 1967; and Farrell, 1974. The plates were incubated at 37° C. in air containing 20 % CO_2 and were examined every 2 days for 10 days. Suspected brucella colonies were provisionally identified by slide agglutination.

Guinea-pig inoculation

Milk ring test positive samples were inoculated into guinea-pigs, but MRT-negative milk samples were inoculated only twice yearly, unless from herds which had previously been MRT-positive. The centrifuged deposit (1400 *g* for 30 min) from 100 ml. of milk was emulsified in the cream layer and 2 ml. inoculated intramuscularly into the thigh of a guinea-pig. When the guinea-pig was killed

Table 1. *The incidence of brucella-infected producer–retailer herds in North Lancashire in the period* 1965–1972

Year	Area I	Area II	Total
1965	36/210* (17)	114/484 (24)	150/694 (22)
1966	30/201 (15)	82/479 (17)	112/680 (16)
1967	29/255 (11)	107/565 (19)	136/820 (17)
1968	33/336 (10)	113/546 (21)	146/882 (17)
1969	19/220 (9)	95/510 (19)	114/730 (16)
1970	13/281 (5)	93/481 (19)	106/762 (14)
1971	16/353 (6)	67/435 (15)	83/688 (12)
1972	1/208 (0·5)	27/366 (7)	28/574 (5)

* Number of herds excreting/number tested.
Figures in parentheses are percentages.

Table 2. *The biotypes of* Brucella abortus *isolated from untreated milk*

Year	No. of herds excreting	No. typed	Biotype			More than one biotype excreted
			1	2	4, 5 or 9	
1965	150	133	110 (83)	11 (8)	19 (14)	7
1966	112	110	86 (78)	12 (11)	17 (15)	4*
1967	136	135	93 (69)	14 (10)	32 (24)	4
1968	146	137	101 (74)	18 (13)	25 (18)	7
1969	114	108	71 (66)	11 (10)	35 (32)	8*
1970	106	104	70 (67)	13 (13)	32 (31)	11
1971	83	80	56 (70)	9 (11)	21 (26)	5
1972	28	25	22 (88)	5 (20)	2 (8)	4

* Including one herd excreting three different biotypes.
Figures in parentheses are percentages.

6 weeks later the spleen and deep inguinal gland adjacent to the site of inoculation were removed and cultured for brucella organisms on 5 % blood agar containing 100 μg./ml. of cycloheximide.

Identification and biotyping

The biotype of each strain of *B. abortus* was determined by the criteria of Alton & Jones (1967): (1) CO_2 dependence; (2) H_2S production on serum-dextrose agar; (3) sensitivity to basic fuchsin and thionin; (4) agglutination with *B. abortus* and *B. melitensis* monospecific sera; (5) sensitivity to *B. abortus* bacteriophage, strain Tblisi.

RESULTS

The incidence of herds excreting brucella organisms in milk was higher in Area II (flying herds) than in Area I (self contained herds) in each of the 8 years of the investigation. *B. abortus* was isolated from the milk of 22 % of all producer–retailer herds in 1965, the incidence declining steadily to 12 % in 1971, then falling sharply to 5 % in 1972 (Table 1). This sudden decrease was more pronounced in Area I, which contributed only one of the 28 herds excreting brucellas in 1972.

In 1965, 83 % of the infected herds were excreting *B. abortus*, biotype I, and 8 % excreting biotype 2 (Table 2). Since 1965 the incidence of biotype 2 has not altered significantly whereas the incidence of biotype 1 fell steadily until 1969, when it was responsible for 66 % of infections. This was accompanied by an increase in the incidence of infections with biotypes 4, 5 and 9, from 14 % in 1965 to 32 % in 1969; biotype 4 has never contributed more than 1 % of the infections for any year and has been included with biotypes 5 and 9 mainly for convenience. Biotypes 5 and 9 would previously have been described as 'British Melitensis'. After 1969 the incidence of these biotypes fell to 8 % whilst the incidence of biotype 1 increased from 66 % to 88 % (Table 2).

DISCUSSION

Cattle are usually infected with *Brucella* by ingestion or inhalation of contaminated material. As a source of contamination the infected pregnant cow is especially dangerous during parturition when large numbers of organisms are discharged in the amniotic fluids, placenta, and fetus, and post partum when vaginal discharges containing large numbers of organisms are present for several weeks. In many infected cows the organism becomes localized in the udder and brucella organisms are excreted in the milk.

Leech and his colleagues (1964) estimated that in 1960–61 25,000 to 30,000 (25 to 30 %) of British dairy herds were infected with *Brucella abortus*, and that 2 % of dairy cows were infected, approximately 50 % (36,500) of which were excreting the organism in their milk.

Short-term control measures were evolved before the introduction of a programme for the eradication of brucellosis. The clinical manifestations of the disease in cattle were controlled with brucella vaccines, S-19 and 45/20, thus reducing the all too familiar 'abortion storms', with great economic advantage to the farmer, but the problems of udder infections and, to a lesser degree of infertility, have remained. The transmission of brucellosis from infected cattle to man has been contained by pasteurization of milk, and where this was not feasible, by the restriction of the sale of brucella-infected, untreated milk. It must be emphasized however, that the risk to those in occupational contact will remain until the disease in cattle is eradicated.

In 1966 the Ministry of Health indicated that existing legislation should be vigorously applied against the sale of brucella-infected, untreated milk (Ministry of Health Circular 17/66). It was suggested that untreated milk supplies should be examined monthly, whenever possible, for the presence of brucella organisms and the sale of infected milk restricted by compulsory pasteurization.

In the North of Lancashire during 1959, Robertson (1961) found that 158/842 (19 %) producer–retailer herds were infected, a figure comparable with our findings for 1965. Despite the increased surveillance recommended by the Ministry and the announcement of the proposed eradication programme (Hansard, 1966) the decline in the incidence of brucella infection in these herds was slight, for in 1969 114/730 (16 %) of herds were producing for sale brucella-infected, untreated

milk. During the time covered by this report there was intense activity by the producer-retailers to identify the infected animals within their herds and these animals were then sold in the open market and introduced infection into other herds, thereby increasing the dissemination of the disease both within the region under investigation and elsewhere in the United Kingdom. This problem was appreciated by Henderson (1969) who described the practice of selling brucella-infected cattle in the open market in Worcestershire.

In Lancashire one County Borough rigorously excluded all brucella-infected raw milk by allowing only ring test negative milk to be sold within its boundaries. The farmers had the ring test positive, and thus potentially infected, animals identified by laboratory tests and proceeded to sell ring test negative milk within the County Borough and ring test positive milk in the contiguous municipal boroughs and rural areas.

In April 1967, the voluntary Brucellosis (Accredited Herds) Scheme was launched with the aim of identifying and registering those herds which could be used as sources of brucella-free replacement stock for other herds.

In April 1970, the replacement Brucellosis Incentives Scheme was introduced. To qualify for entry on the national register, a herd has to have three consecutive negative blood tests at four-monthly intervals.

In November 1971, eradication of brucellosis was started in three main areas of Great Britain with a programme of compulsory blood testing of all herds not already in the voluntary schemes.

Once the Brucellosis Incentives Scheme was introduced and the eradication areas and extension zones were specified the attitude of stock owners appeared to alter. The region described in this communication is not an initial eradication area, but a large part of Area I, which contains mainly large self-contained herds, is included in the extension zone for the next phase to be started in November 1973. These factors have undoubtedly contributed to the large decline in infections already described.

The distribution of the biotypes of *B. abortus* shows that the frequency of biotype 1 infection fell during the period 1965 to 1969 but with a corresponding increase in infection with biotype 4, 5 and 9 (Table 2), until in 1969 these biotypes caused 32 % of the brucella infections in this region.

It is significant that this change in the epidemiological picture occurred at a time when infected animals were being identified and their removal from herds actively encouraged, but without adequate powers to control their movement or to demand their slaughter.

Since 1970, the reversal of this trend has coincided with the acceptance of the Brucellosis Incentives Scheme by the producer-retailers in North Lancashire, with the slaughter of infected animals and their replacement by brucella-free stock.

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J. Hyg., Camb. (1974), **73**, 15
Printed in Great Britain

On the use of contour maps in the analysis of spread of communicable disease*

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(*Received* 17 *October* 1973)

SUMMARY

The co-ordinates of the dwellings where cases of variola minor (alastrim) occurred during a small epidemic were used in a worked example of contour mapping of disease spread. The contoured variable was the date of onset, relative to an arbitrary base date, of the case introducing the disease into each of twenty-two households. Three contour maps prepared with slightly different computer programmes or dates exhibited similar concentric loops whose centres were close to the first infected household. The average rate of spread of the disease was estimated by regression of the number of days to onset of the first case in the household on the average distance from an arbitrary origin to the relevant contour line. The calculated average rate of spread was 1·22 metres per day. An additional map was contoured using the cumulative number of cases as the contoured variable, relative to the onset of the example epidemic.

INTRODUCTION

Contour maps have long been used for pictorial representation of the relations between geographical co-ordinates of given places and the corresponding values of a dependent variable. Application of contour maps to metereological, geological, topographical and geographical topics are well known. Fairly recently, contour maps have been used in Ecology, for instance, for studying geographical variation of characteristics of animal and plant species (Adams, 1970; Kiester, 1971). More rarely, contour mappings of disease have dealt with geographical locations of endemic foci (Hopps, 1969). It seems that the dynamics (time and space distributions) of epidemic spread of diseases transmitted from person to person have not been examined by contour mapping, in spite of the interesting possibilities of this approach. An attempt was made to analyse, by contour mapping, the spread of variola minor during a small epidemic. The results are reported as a worked example, in the hope that they may stimulate application to larger outbreaks with various modes of disease spread.

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Table 1. *Basic data for the contour-map analysis of the spread of variola minor in Vila Guarani*, 1956

Geographic co-ordinates of dwellings with cases* (x cm.)	(y cm.)	Identification number of the household with cases	Number of cases in the household	Cumulative number of cases†	Date of onset of the first case in the household‡
7·5	6·7	1	1	1	18
7·5	6·7	2	4	5	40
7·5	6·7	3	1	6	56
7·6	4·7	4	2	8	59
7·4	9·2	5	3	11	84
8·1	5·5	6	3	14	72
11·4	8·5	7	2	16	109
10·9	3·7	8	1	17	93
17·7	5·9	9	1	18	93
12·5	9·0	10	4	22	111
3·8	4·1	11	2	24	108
8·5	5·2	12	1	25	94
4·9	13·7	13	11	36	105
4·8	4·1	14	6	42	110
0·7	12·7	15	2	44	111
6·8	7·3	16	1	45	112
10·6	4·6	17	1	46	113
5·6	13·9	18	1	47	117
2·5	4·3	19	2	49	124
4·4	7·8	20	3	52	138
4·4	7·8	21	1	53	160
4·4	7·8	22	1	54	178

* Actual measurements on a map at a scale of 1 in 4270.

† Relative to the case introducing the disease into the district.

‡ The date of onset has been converted to the number of days from an arbitrary base of 1 March 1956.

MATERIALS AND METHODS

Epidemic data

The basic data for the application of the example are the published time and space distributions of cases of variola minor (alastrim) during the epidemic occurring, in 1956, in Vila Guarani, a semi-rural school district of the city of São Paulo, state of São Paulo, Brazil (Rodrigues-da-Silva, Rabello & Angulo, 1963; Angulo, Rodrigues-da-Silva & Rabello, 1968). The dates of onset were thoroughly investigated and even the chain of contagion could be reconstructed in detail with an unusually high degree of credibility (Angulo, Rodrigues-da-Silva & Rabello, 1968). The dwellings with one or more cases were located on a map prepared from an aerophotogrammetric plot, after actual visits to these dwellings.

Manipulation of data for computation

The data included in the published epidemic curve, chain of contagion and geographical distribution of dwellings with cases (Rodrigues-da-Silva *et al.* 1963; Angulo *et al.* 1968) were manipulated for application of computer-plotting pro-

cedures. For convenience of calculations, the calendar dates were converted to the number of days from an arbitrary base of 1 March 1956. That was the month when variola minor was introduced into the essentially closed community where the example epidemic occurred (Rodrigues-da-Silva *et al.* 1963). The original scale of the aerophotogrammetric plot of the Vila Guarani district, 1 in 2000, was reduced in the published map (Angulo *et al.* 1968) to 1 in 4270. A grid with squares of 1 cm. side was superimposed on the reduced map. The vertical and horizontal co-ordinates of the centre of each dwelling with one or more cases were measured and they appear in Table 1. This table also shows the household identification number, the number and cumulative number of cases occurring in each household and the converted date of onset of the first case in the household, hereinafter called the date of onset.

The fact that, in two instances, three households were living in a single house, although separated by internal partitions, is recognized in the original publication (Angulo *et al.* 1968) as well as in the present paper's Figs. 1 and 3. In these figures, the date of onset appears separately for each household. On the other hand, Fig. 2 presents a single averaged value for the three households in the above two instances (Households 1, 2 and 3 and Households 20, 21 and 22). The date of onset is the value contoured in each of Figs. 1 to 4, while the value contoured in Fig. 5 is the cumulative number of cases (Table 1).

The dependent variable (date of onset) together with the independent variables (the co-ordinates) were used as input to the computer-contouring package used by Splaine, Lintott & Barclay (1970). The IBM Computer Programme 'Numerical Surface Techniques and Contour Map Plotting' (see the Appendix to the present report) was employed in an IBM computer fitted with an IBM 1627 model 2 Plotter. Later, contouring by the same computer, using the Calcomp G.P.C.P. programme package (see Appendix) and a Calcomp Plotter, was tried for comparison purposes. Finally, the cumulative number of cases was used as the input to the Calcomp programme.

RESULTS

Contour mapping

Four contour maps were produced by the computer. Fig. 1 shows the contour map produced by using the contouring package used by Splaine *et al.* (1970). It exhibits three almost concentric loops in the centre but no readily discernible pattern outside this area. Fig. 2 exhibits the contour map produced by the same computer using the Calcomp G.P.C.P. package. This package contours to the specified map boundary regardless of sparseness of data. Also, the dates of onset in Households 1, 2 and 3 are shown as a single averaged value. The same applies to Households 20, 21 and 22. As said before, each of these groups of three households lived in a single house with internal partitions separating the house into three household dwellings. Fig. 3 shows the contours obtained with the same G.P.C.P. package, but with a set of printing instructions somewhat different from that used for producing the map from Fig. 2.

Fig. 5 shows the contours obtained with the Calcomp G.P.C.P. package, when

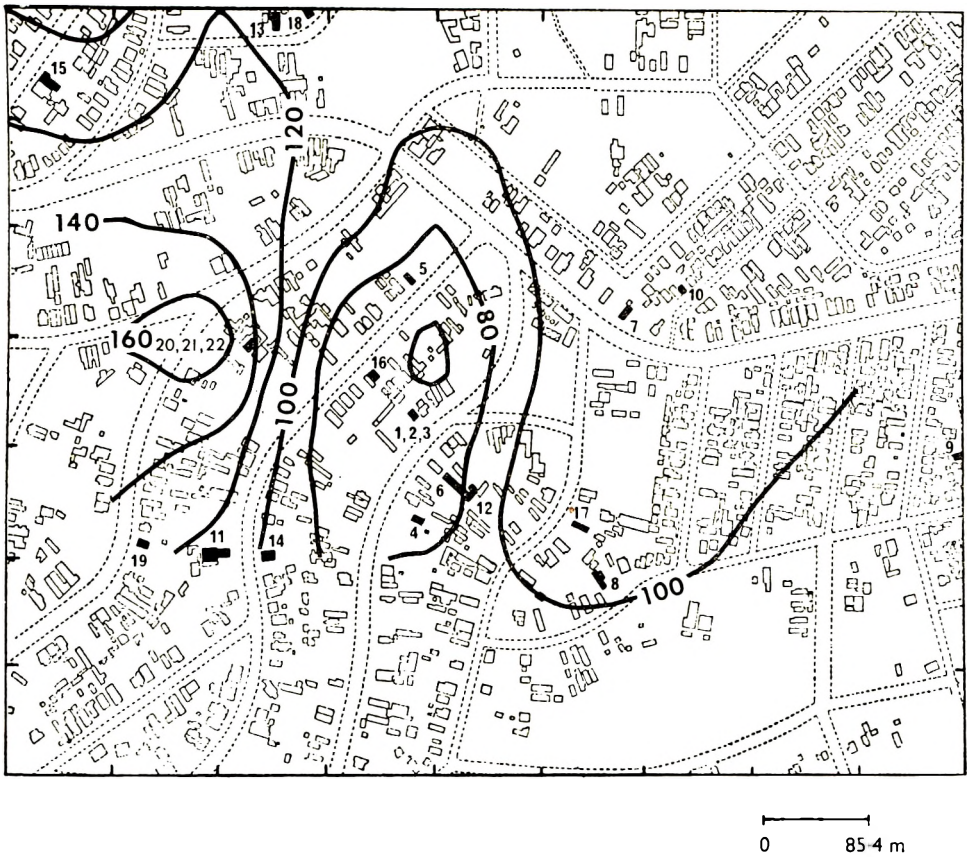

Fig. 1. Contour lines superimposed on an aerophotogrammetric map of Vila Guarani, a school district where an epidemic of variola minor occurred in 1956. The blackened polygons are the dwellings where cases occurred and their numbers correspond to those in Table 1. The contour value of each household with cases is that appearing in the last column of Table 1. The computer programme was the IBM N.S.T.C.M.P. package.

the dependent variable was the cumulative number of cases (Table 1). This number of cases is an indication of the progress or course of the epidemic. The contouring pattern is essentially the same as that provided by the cumulative time intervals to onset of the first case of the epidemic (Figs. 1–4). It should be mentioned that the later plots (those from Figs. 3 and 5, for instance) were generated by increasing the number of neighbouring data points used for the determination of mesh point values, thus increasing the degree of smoothing of the original data.

In a few places on the maps obtained (e.g. Household 8 in Fig. 1), a contour can be seen to lie on the 'wrong' side of the position of the household dwelling. Such occurrences are due to the averaging and smoothing procedures employed in the programmes and in no case do they invalidate the general interpretative picture produced. With only 22 data points available, it is not surprising that procedures which differ in detail should produce localized differences in graphical

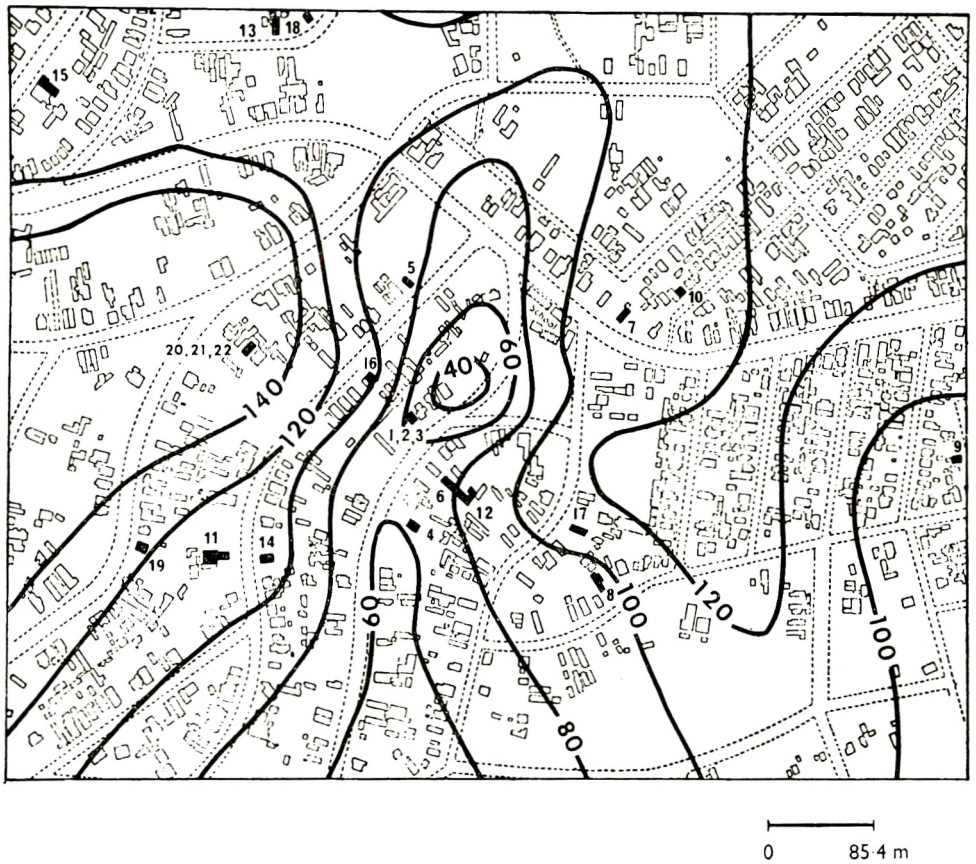

Fig. 2. Same as in Fig. 1, except that the computer programme is the Calcomp G.P.C.P. package. Also, the contour values of Households 1, 2 and 3 were averaged since they lived, separated by internal partitions, in a single house. The same applies to Households 20, 21 and 22.

interpretation. It is not the purpose of this paper to analyse the relative merits of these programmes, but merely to point out that, in general, the maps follow the same over-all pattern. While contour maps display interesting general trends, caution must be exercised in drawing specific conclusions from the detailed configuration of the contours.

Estimation of the rate of disease spread

It seems practicable to estimate an average rate of spread of variola minor from the contour map of say, Fig. 3. On this contour map, an arbitrary origin was chosen, nominally at the centroid of the innermost loop. From this centroid, sixteen radial lines were drawn with uniform angular spacing. The distances (in cm.) from the origin to the contour line were then read off by ruler, along each of the radial lines, provided that the contour value (number of days to onset) continued to increase along each radial line. All such measured distances are shown in Table 2. These measurements may be considered as estimates of the average radius of each particular contour of date of onset, in the case of a closed

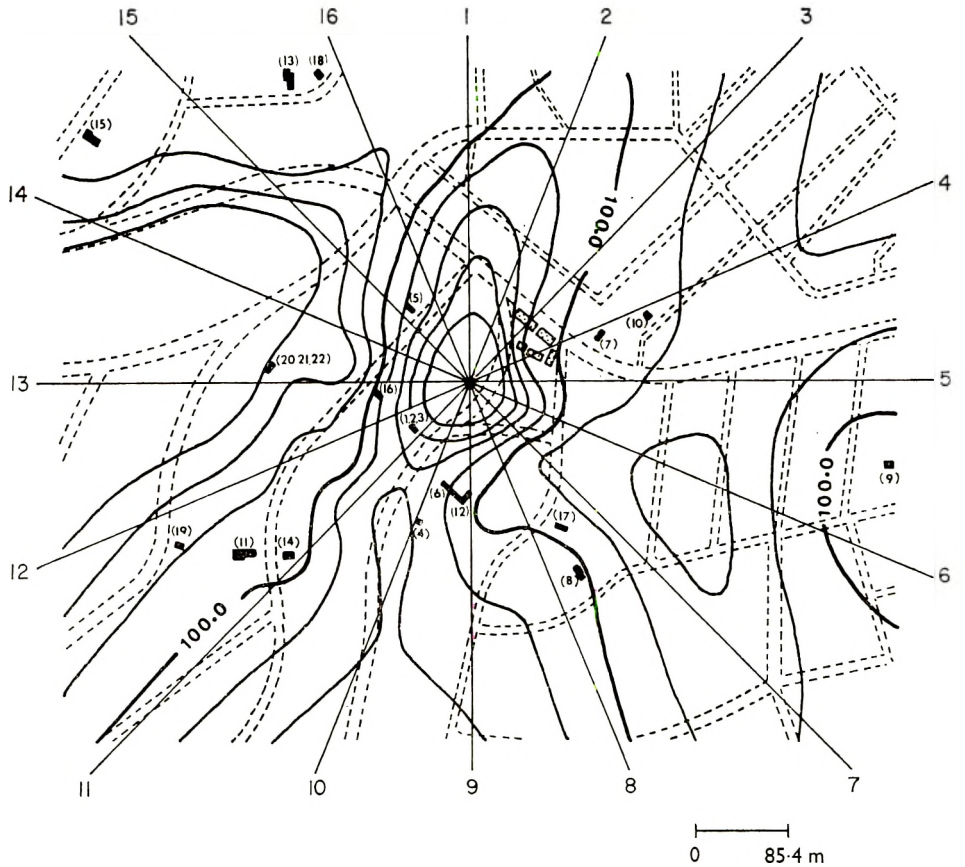

Fig. 3. Same as in Fig. 2, except that the computer programme had different instructions for plotting the data and more detail was obtained. Also, the contour values are not averaged for either Households 1, 2 and 3 or Households 20, 21 and 22. From an arbitrary centre, radial lines have been drawn to estimate the rate of spatial spread of variola minor according to the procedure described in the text.

contour. Average distances (in cm.) were then plotted against the number of days to onset, at intervals of 20 days (Fig. 4). A reasonably straight line was obtained. It was arbitrarily chosen to regard this average radius as the mean distance covered by the disease in the number of days indicated by the numerical value attached to the contour line. The precise definition breaks down in the case of open contours. Nevertheless, it may be assumed that the distances so measured are in some way representative of the rate of spread. Under these circumstances, the reasonably straight line obtained in Fig. 4 suggests a constant linear rate of spread.

Using the data in Table 2, the equation of the regression line for predicting the distance from the origin (Fig. 3), for a given time in days, is

$$\text{distance in centimetres} = 0{\cdot}1874 + 0{\cdot}02861 \times \text{time in days.}$$

Thus, the average rate of spread of variola minor was 0·0286 cm. per day on the already published map (Angulo *et al.* 1968) and on Fig. 3. For a more suitable

Table 2. *Estimation of the average rate of spatial spread of variola minor in Vila Guarani, 1956, by distances measured from an arbitrary origin to intersection with contour lines along radial lines**

Radial line no.†	Contour line value							
	20	40	60	80	100	120	140	160
1	1·35	2·35	3·40	4·15	4·90	—	—	—
2	1·30	1·80	3·05	4·90	—	—	—	—
3	0·95	1·20	1·45	2·05	3·90	7·35	—	—
4	0·80	0·95	1·15	1·35	2·20	4·95	7·75	—
5	0·85	1·05	1·25	1·55	2·10	3·80	—	—
6	0·90	1·15	1·50	1·80	2·10	2·60	4·05	—
7	0·95	1·20	1·40	1·60	1·80	2·30	—	—
8	0·90	1·15	1·30	1·60	1·90	—	—	—
9	1·00	1·25	1·50	1·90	—	—	—	—
10	1·05	1·40	1·95	—	—	—	—	—
11	1·10	1·45	1·95	4·05	4·85	—	—	—
12	1·00	1·35	1·65	2·05	2·50	4·50	7·50	—
13	0·85	1·15	1·40	1·65	1·95	2·30	3·30	4·55
14	0·85	1·10	1·40	1·65	2·00	2·40	2·90	3·85
15	0·90	1·20	1·50	1·95	2·55	3·10	3·75	—
16	1·05	1·40	1·95	2·70	3·50	4·70	—	—
Mean	0·9875	1·322	1·737	2·330	2·788	3·800	4·875	4·200

* Figures in the columns correspond to centimetres actually measured on the published map of Vila Guarani (Angulo, Rodrigues-da-Silva & Rabello, 1968). This map was reduced from an aerophotogrammetric plot. See Fig. 3.

† From Fig. 3. The contour-line values correspond to dates of onset of the first case in the household, after conversion of the calendar date to a number of days from base of 1 March 1956. March was the month when variola minor was introduced into Vila Guarani (Rodrigues-da-Silva, Rabello & Angulo, 1963).

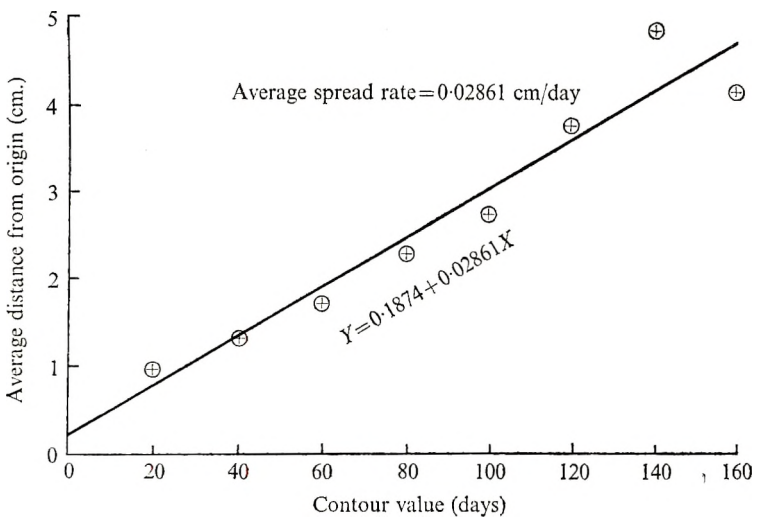

Fig. 4. Regression of the average distance from the arbitrary origin appearing in Fig. 3 on the contour values of the households in the last column of Table 1.

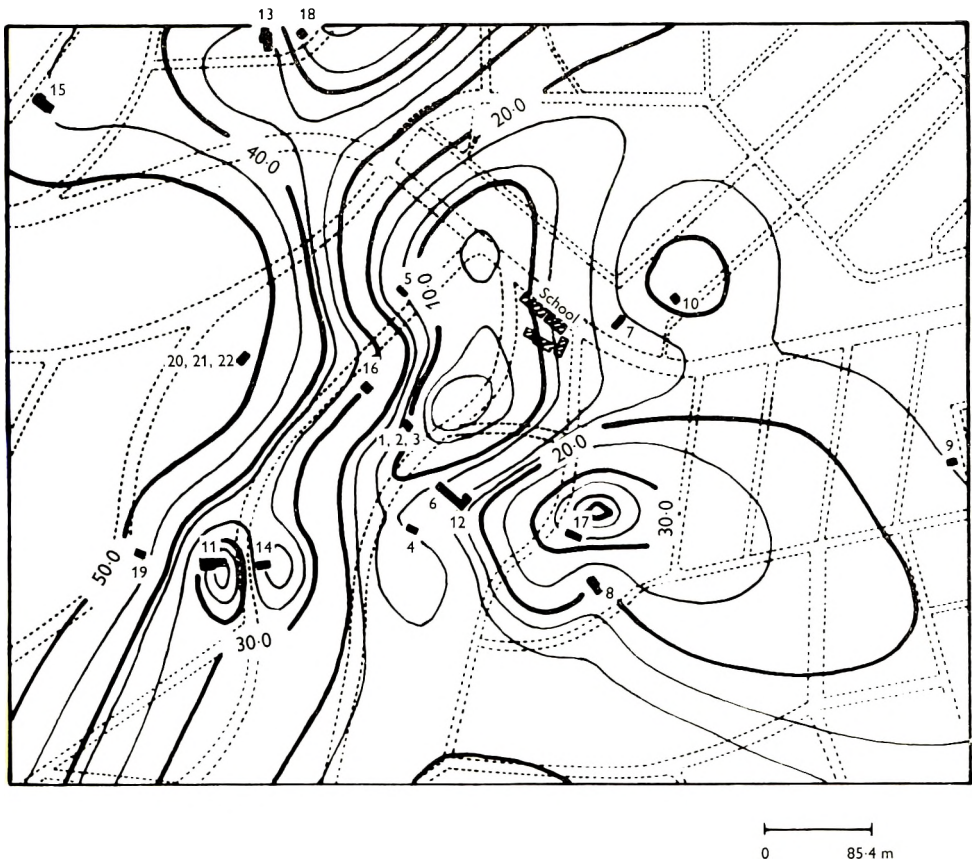

Fig. 5. Same as in Figs. 1 to 4, except that the contoured variable is the cumulative number of cases (Table 1). The computer programme is the Calcomp G.P.C.P. package. The contoured value for the building housing Households 1, 2 and 3 is the cumulative number of cases for Household 3. For the building housing Households 20, 21 and 22, the contoured value is the cumulative number of cases of Household 22.

presentation of these results, they have been corrected according to the scale of the map (1 in 4270). Thus, the estimated average rate of spread should read: 1·22 metres per day.

DISCUSSION

The results obtained by the use of contour mapping are presented only as a worked example, with no claim as to statistical rigor nor as a definitive formulation of the problem of estimation of the rate of spread of a communicable disease. In this regard, the example epidemic only provided small numbers. On the other hand, the procedure devised for estimating the average rate of spread provides an objective measure of this rate, based on a scattered set of observations.

Some loops are in spatial association with attacked dwellings, while no definite loop is found close to other dwellings with cases. This is seemingly so because, when contouring data which have considerable variability about some underlying pattern, one is bound to find sets of closed contours associated with some, at

least, of the data points. This behaviour is inherent since all points do not lie on the 'underlying' surface pattern. Thus, no special significance should be attached to loops associated with single data points. Differences in dates of onset between households occupying different parts of the same building are a serious embarrassment to the contouring method of analysis. The latter method, of its very nature, cannot make much sense from such data.

If the number of cases in each household was approximately the same and if the intervals between successive dates of onset were approximately constant, the contouring programme would not find any significant difference between data sets formulated for date of onset on the one hand, and for cumulative number of cases on the other. The data from Vila Guarani conform in some respects with the above hypotheses, so it is not surprising to note general similarities between the respective contour maps. The differences between the maps are merely a measure of the random departures of the individual data items from the hypotheses.

With reservations due to the limitations of the example data and procedures, it may be said that the use of contour mapping for studying the spatial spread of variola minor disclosed interesting general trends. For instance, a fairly uniform spread of the disease from a central point. This perhaps justifies the kind of calculations presented above. However, the detailed shapes of the contours are a function of a mathematical procedure based on only twenty-two observations (households). Therefore, no specific meaning should be attached to any one small region of the contour map, particularly to loops associated with a single data point. With these reservations in mind, it may be pointed out that the centre of the concentric loops, evident in all three first contour maps obtained, is no more than 65 m. distant from the location of the first infected household.

It is known that, at least partially, variola minor spread among the students at the school (Angulo, Rodrigues-da-Silva & Rabello, 1964). Reconstruction of the chain of contagion and inspection of the dates of onset of cases in households yielded a highly suggestive evidence of the role of the district school as a nodal centre for the spread of variola minor in Vila Guarani district (Angulo *et al.* 1968). However, the close proximity of the 'centre' to the location of the school (no more than 100 m.) is believed to be fortuitous. In effect, there is no reason to suppose that the date of infection of a student at the school could be correlated with the distance between the infected student's home and the school. It must be concluded that, if the fairly uniform outward spreading pattern shown by the contour maps has any meaning at all, the pattern must largely represent the spread of the disease by contacts other than those at the school. In this regard, the epidemic in Vila Guarani was found to consist of summation of smaller, almost independent outbreaks occurring in sub-areas, the dwellings of the households where the disease was introduced by a single member (Angulo, Rodrigues-da-Silva & Rabello, 1967, 1968).

If the school was the sole means of spread of the disease, a formless jumble of contour lines would be expected. If house-to-house was the sole means of spread, fairly regular concentric contours over the whole map would be expected. For a mixture of the two means of spread, a pretty irregular pattern would be

expected, but with some concentric contours present. The patterns obtained for the Vila Guarani district are consistent with this expectancy.

It is felt that contour maps drawn by the procedure used in the present study are interesting from an interpretational point of view for the Vila Guarani district. The value of such maps for epidemic areas with very different patterns of children attending school remains to be investigated. Estimation of the rate of spread through contour mapping suggests that while variola minor was spreading in Vila Guarani, its linear rate of spread was fairly uniform. Although the procedure used to obtain these results seems to be reasonable, the validity of its estimates is unknown.

Meyers (1949) found very variable and slow rates for the spread of measles from the origin to the various other areas of a health-centre district of New York City. In his intuitive study, this worker employed distances from the centre of the area where the epidemic started and the time to reach a peak in the various other areas of the district. Meyers also suggested that the elementary schools were important in the spread of the epidemic. Slow rate of geographic spread of measles was also noted by Stocks (1930).

One of the authors (J.J.A.) gratefully acknowledges the facilities provided by Dr W. Buell Evans.

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APPENDIX

The IBM Computer Programme 'Numerical Surface Techniques and Contour Map Plotting'

The first stage in the IBM computer programme estimated the value of the date of onset for every intersection of a square grid within the required boundaries of the published map. For each 1 cm. square enclosed by adjacent grid points, a plane was constructed in the space defined by the three dimensions: longitude, latitude and date of onset. If there were any observations within a 1 cm. square, the plane was forced to pass through the centroid of those observations. The attitude of the plane was then chosen to give the least-squares fit with the nearest observation in each octant outside that square. The relevant value of the date of onset was inversely weighted by distance from the square. If there was no observation within the 1 cm. square, the plane was fitted by least squares to the above eight external observations without being forced through an internal centroid of observations. When the equation to the fitted plane had been obtained, the programme calculated the implied values of the date of onset in each corner of the 1 cm. square of the grid. The procedure was repeated until the whole map area had been dealt with. Thus, except at the periphery, where there were only two values and the corners, where there was only one value, there were four calculated values for each grid intersection point. These values were averaged to obtain a single value for subsequent use.

In a second stage, the programme calculated a path for each required contour within each square in turn. It then issued the necessary instructions to the X–Y plotter for drawing the contour lines. Each contour path was calculated as follows. Any contour line within a square usually intersected with two of the square sides. These intersection points were found by linear interpolation between adjacent corners of the square. The two relevant adjacent 1 cm. squares were then inspected to locate the next nearest points of intersection of the named contour with a grid mesh line. The programme had thus four points to consider. A circle was fitted to the first three points of the set and a second circle was fitted to the last three points of the set. The two circles intersected within the original square and an intermediate curve was constructed within that square. It was a weighted mean of the two circles. The X–Y plotter plotted this intermediate curve. If any other required contours lay within that square, they were dealt with similarly. The whole procedure was then repeated until all grid squares had been dealt with. Contour values were printed at suitable intervals. Towards the edges of the mapped area, contour plotting was inhibited when the number of observations lying outside a given grid square fell below a critical value.

This description of the essential details of the IBM programme should convey some idea of the trouble to which it is necessary to go in order to achieve a sound, graphical interpretation of a number of discrete data points. The alternative programme which was available (Calcomp G.P.C.P.) used a set of procedures which were different in detail, but which led to similar contour maps.

Calcomp G.P.C.P. Contour Generation

The programme initially generates function values over a user-defined rectangular grid using an approximating process that consists of two basic operations:

(*a*) tangent plane or gradient determination; and

(*b*) the extension of this information to generate grid value.

Tangent plane generation for each input point begins with the selection of n (usually $n = 8$, but may be user-controlled) neighbouring data points that are closest to the point in question. The plane must then pass through the value of the function at the control point and be of such orientation as to minimize the sum of the angles that it makes with the vectors or lines to the neighbouring points. These angles are, of course, weighted by a function of distance of the neighbouring point to the control point. In this manner, a tangent plane (or gradient) is evaluated for each input (control) point.

Grid generation, like tangent plane generation, begins by the selection of the n neighbouring data points (now with gradients) closest to the mesh point in question. The gradient or tangent plane information for each point is evaluated at the mesh point location and the evaluation is weighted inversely on the basis of the distance from the mesh point to the various data points. The surface approximation process is complete after this process has been applied to all mesh points.

Before drawing the contours, the G.P.C.P. package refines each grid cell by dividing each cell into a subgrid. A third order interpolation in X and Y defines the contours crossing each grid cell but at the same time preserves the gradient of the function across cell boundaries.

J. Hyg., Camb. (1974), **73**, 27
Printed in Great Britain

Statistical analysis of data bearing on the number of particles required to form a plaque

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(*Received* 26 *October* 1973)

SUMMARY

Methods of statistical analysis are presented for one or more dilution series experiments where the quantity of interest is the number of virus particles required to infect a cell. These methods are illustrated on several data sets drawn from the literature. Data from seven series, which have been used to support a two-particle model in the literature, are here shown to reject such a model decisively, whereas fifteen other experiments are found to be in excellent agreement with a one-particle model.

INTRODUCTION

A fundamental question that has been of interest in virology for some time is the number of virus particles required to infect a cell. A straight line relationship between concentration of virus innoculated and number of plaques observed is usually taken to be evidence for a single particle (Dulbecco, 1952; Dulbecco & Vogt, 1954; Khera & Maurin, 1958; Kjellén, 1961; Cooper, 1961; Boeyé, Melnick & Rapp, 1966). If two particles are required, the relationship between plaque count and dose is quadratic. In general, if h particles are required to form a plaque, the number of plaques is proportional to the hth power of the concentration (Dulbecco & Vogt, 1954; Cooper, 1961; Boeyé *et al.* 1965). Where evidence concerning linearity or non-linearity has been presented, it appears to have been assessed by examining visually graphs of concentration versus observed plaque count, or by presenting estimates of h. No quantitative or statistical assessment of the strength of the evidence in this regard appears to have been made.

The effort and expense involved in a thorough statistical analysis will usually be negligible in comparison with that required to obtain reliable data. It therefore seems only reasonable that, when observations have been carefully made, a complete analysis should be undertaken in order to extract the maximum amount of information from them.

It is the purpose of this paper to present appropriate methods of statistical analysis. The necessity of a statistical analysis is demonstrated on data which have been taken to support a two-particle model in the literature but which are here shown to reject such a model decisively. Hence these methods can sometimes detect departures from the predicted relationship that are not apparent from visual inspection alone.

THE MATHEMATICAL MODEL

Suppose that there are k successive dilutions of an initial viral concentration by a dilution factor d, giving $k+1$ concentrations in proportion to $1:d^{-1}:d^{-2}:\ldots:d^{-k}$. The assumptions usually made are that it requires h particles to infect a cell; that the initial concentration of virus particles is not too great; and that the virus particles are randomly distributed among the cells on the plate. It can then be shown that the theoretical or expected plaque count at dilution level j (concentration d^{-j}) will be

$$\nu(d^{-j})^h = \nu\theta^j,$$

where ν is the expected number of plaques arising from the undiluted suspension, and $\theta = d^{-h}$ (Dulbecco & Vogt, 1954; Cooper, 1961; Boeyé *et al.* 1966). Furthermore, it follows *from the same assumptions* that the actual plaque count, which is subject to random fluctuations about this expected value, will follow a Poisson distribution (Reid, Crawley & Rhodes, 1949; Kjellén, 1961; Cooper, 1961; Boeyé *et al.* 1966; Fisher, 1970, 54–63). The probability of observing y_j plaques at level j is then

$$(\nu\theta^j)^{y_j} \exp(-\nu\theta^j)/y_j!.$$

This model was used by Alling (1971). If there are n_j separate flasks or plates at dilution level j, then y_j will be used to denote the total number of plaques on all n_j plates, and $\nu\theta^j$ will be replaced by $n_j\nu\theta^j$.

The statistical analysis presented in the following sections flows from the above model. The quantity of interest is h, the number of particles required to form a plaque. The quantity ν is not usually of interest, and is eliminated from the analysis by the technical device of conditioning on the total plaque count over all dilutions. The analysis is then based on the fact that the maximum likelihood estimate of h has approximately a normal distribution. For justification and mathematical details see Kalbfleisch & Sprott (1974).

STATISTICAL ANALYSIS OF A SINGLE SERIES

Define y_j, n_j, d, h, and $\theta = d^{-h}$ as above, and let $\log d$ denote the natural logarithm of d. The following quantities enter into the analysis:

$$\left.\begin{aligned} X &= y_0 + y_1 + y_2 + \ldots + y_k; \\ T &= \quad y_1 + 2y_2 + \ldots + ky_k; \end{aligned}\right\} \tag{1}$$

$$\left.\begin{aligned} B &= n_0 + n_1\theta + n_2\theta^2 + \ldots + n_k\theta^k; \\ A &= \quad n_1\theta + 2n_2\theta^2 + \ldots + kn_k\theta^k; \\ D &= \quad n_1\theta + 2^2n_2\theta^2 + \ldots + k^2n_k\theta^k; \end{aligned}\right\} \tag{2}$$

$$S = -\left(T - X\frac{A}{B}\right)(\log d); \tag{3}$$

$$I = X(DB - A^2)(\log d)^2/B^2. \tag{4}$$

Table 1. *Analysis of plaque counts from Boeyé et al.* (1966)

Isolate	Dilution level 0	1	2	$\hat{h}$	$\hat{I}$	u ($h = 2$)	u ($h = 1{\cdot}8199$)
1	122	10	2	2·0280	19·63	0·124	0·922
2	176	10	4	2·1085	25·18	0·544	1·448
3	170	19	2	1·9116	32·34	−0·503	0·521
4	266	40	5	1·6755	70·54	−2·725	−1·213
5	264	38	4	1·7303	64·88	−2·172	−0·722
6	306	42	3	1·8076	67·63	−1·582	−0·101
7	186	22	2	1·8889	36·58	−0·672	0·417

The first step in the analysis is the computation of $\hat{h}$, the maximum likelihood estimate (M.L.E.) of h. This is the value of h which is best supported by the data in the sense that, when $h = \hat{h}$, the probability of the observed plaque counts is as great as it possibly can be under the model. The estimate $\hat{h}$ may be obtained by solving the equation $S = 0$ using the procedure described in the Appendix. For discussions of maximum likelihood estimation, see Finney (1964, p. 80) and Kempthorne (1969, p. 167).

If X, the total plaque count over all dilutions, is not too small, then the maximum likelihood estimate $\hat{h}$ will have approximately a normal distribution with mean h and variance $\hat{I}^{-1}$, where $\hat{I}$ is the value of I computed from (4) using $h = \hat{h}$. Hence the quantity u defined by

$$u = (\hat{h} - h)\sqrt{\hat{I}} \tag{5}$$

will have approximately a standardized normal distribution (a normal distribution with mean 0 and variance 1), for which tables are readily available. In order to determine whether a proposed value of h is consistent with the data, the corresponding u-value is computed from (5) and is compared with the tables. An improbably large or small (negative) value of u provides evidence against the proposed value of h. An approximate 95 % confidence interval for h is given by $\hat{h} \pm 1{\cdot}96/\sqrt{\hat{I}}$, this being the set of h-values for which u lies within the central 95 % of the standardized normal distribution.

Example. Columns 2, 3 and 4 of Table 1 give plaque counts from Boeyé *et al.* (1966, Table 4), which were taken as supporting a two-particle model ($h = 2$). There are three dilution levels ($k = 2$), the dilution factor d is $\sqrt{10}$, and each plaque count is the total over $n_j = 2$ plates. The fifth column gives $\hat{h}$ for each isolate, computed as in the Appendix. Column 6 gives $\hat{I}$, which is computed from (4) with $h = \hat{h}$. Column 7 gives the value of u for each isolate as computed from (5) with $h = 2$. Isolates 1, 2, 3, 6, and 7 are now seen to be consistent with $h = 2$ because the corresponding values of u are reasonable ones (within the central 95 % of a standardized normal distribution). However, isolates 4 and 5 yield u-values which differ from zero by more than 1·96, and therefore contradict the assumption that h is 2.

The last column of Table 1 gives the value of u for each isolate as computed from (5) using $h = 1{\cdot}8199$. These values will be used in the next section.

COMBINATION OF DATA FROM SEVERAL SERIES

Given the results of r dilution series experiments, such as the seven isolates of Table 1, two questions will be of interest. First, one will wish to know whether the data are homogeneous; that is, whether there exists a single value of h which is compatible with the data from all r experiments. Secondly, assuming a common value of h, one will wish to combine the data from the r experiments to give a single estimate of h, or to test some theoretical value such as $h = 2$ in the preceding example.

Combination of the data

The overall maximum likelihood estimate of the common value of h in r experiments can be obtained easily on an electronic computer. However, the following analysis is computationally much simpler, and will give almost identical results provided that the total plaque count X is fairly large in each experiment.

Let the M.L.E. of h in the ith experiment be denoted by $\hat{h}_i$, with approximate variance $\hat{I}_i^{-1}$ as calculated in the last section. An overall estimate of h may be obtained as a weighted average of the individual estimates:

$$\bar{h} = \Sigma \hat{I}_i \hat{h}_i / \Sigma \hat{I}_i. \tag{6}$$

This has approximately a normal distribution with mean h and variance $(\Sigma \hat{I}_i)^{-1}$. Hence the quantity

$$z = (\bar{h} - h)\sqrt{\Sigma \hat{I}_i} \tag{7}$$

has approximately a standardized normal distribution, and may be used to assess an hypothesized value of h on the basis of the combined data from all of the experiments.

Heterogeneity of the data

To test for heterogeneity in the data, compute

$$\chi^2 = \Sigma u_i^2, \quad \text{where} \quad u_i = (\hat{h}_i - \bar{h})\sqrt{\hat{I}_i}. \tag{8}$$

Under the assumption of homogeneity, χ^2 has approximately a chi-square distribution with $r-1$ degrees of freedom, and this distribution is extensively tabulated. An improbably large value of χ^2 would show that there was no single value of h which was compatible with the data from all r experiments.

Example (continued). The values from Columns 5 and 6 of Table 1 may be substituted in (6) to give the weighted average $\bar{h} = 1{\cdot}8199$, with variance

$$(\Sigma \hat{I}_i)^{-1} = (316{\cdot}77)^{-1}.$$

The values $u_1, u_2, \ldots, u_7$, computed from (5) with $h = 1{\cdot}8199$, are given in the last column of Table 1, and their sum of squares is $\chi^2 = 5{\cdot}395$. The value of χ^2 is a very probable one, lying close to the 50% point of a chi-square distribution with 6 degrees of freedom, and hence there is no evidence of heterogeneity among the seven isolates.

To determine whether the combined data of all seven isolates are consistent

with the two-particle model, set h equal to the theoretical value 2. Then (7) gives $z = -3{\cdot}205$. The chance of obtaining a value so far from zero in a standardized normal distribution is less than 0·003, and hence *the combined data from all seven isolates are incompatible with the two-particle model.*

It is perhaps not obvious from a visual inspection of the estimates of h in Table 1 that the combined experiment provides such strong evidence against the theoretical value $h = 2$. In fact, *it has been concluded elsewhere in the literature that these data support the two-particle theory.* This example shows the need for quantitative methods, without which the strength of the evidence cannot adequately be assessed.

Further analysis may be undertaken in an attempt to determine the reason for the departures from the two-particle model. The assumption of a Poisson distribution can be checked statistically and appears to be satisfactory. The possibility of a one-particle model is easily ruled out because $h = 1$ is even more decisively rejected by the data than $h = 2$. Indeed, no integer value of h is compatible with the data. A possible explanation is that generally two particles are required to form a plaque, but there is a small probability that one particle will suffice.

DATA CONFORMING TO A ONE-PARTICLE MODEL

In the preceding example, the dilution factors and numbers of dilution levels were the same in all seven isolates. For a more complex example, data from $r = 15$ dilution series experiments were taken from the following four easily accessible sources:

(1) Dulbecco (1952); (2) Dulbecco & Vogt (1954);

(3) Khera & Maurin (1958); (4) De Maeyer (1960).

The plaque frequencies and dilution factors for these 15 experiments are recorded in Table 2 according to their source. The numbers of plates used are given in parentheses. For instance, in experiment $(2d)$ the dilution factor d was 3, and there were three dilution levels ($k = 2$). At dilution level 0 there were 2 plates with a total of 46 plaques; at level 1 there were 6 plates with a total of 61 plaques; and at level 2 there were 10 plates with a total of 36 plaques. The remaining dilution levels 3, 4, 5, and 6 were not used in experiment $(2d)$.

The second column of Table 3 gives $\hat{h}$ for each of the 15 experiments computed as in the Appendix. The calculations may be performed by desk calculator in a few hours, or by electronic computer in a few seconds. For the latter, all that is required is a routine to evaluate S and I as defined by (3) and (4). The calculations outlined in the Appendix can then be carried out by repeatedly applying this programme.

The third column of Table 3 gives the value of I when $h = \hat{h}$ for each of the fifteen experiments, and column four gives the value of u for each experiment, computed from (5) using the theoretical value $h = 1$. All fifteen values of u lie within the central 95 % of a standardized normal distribution. Hence each individual experiment is compatible with a one-particle model, and this agrees with the conclusions reached in the literature.

Table 2. *Plaque counts and numbers of plates used in fifteen experiments*

Experiment	Dilution level 0	1	2	3	4	5	6	Dilution factor d
(1) *a*	297 (2)	152 (2)	—	—	—	—	—	2
b	112 (2)	124 (7)	—	—	—	—	—	3
c	79 (1)	23 (1)	—	—	—	—	—	3
d	50 (1)	—	12 (1)	2 (1)	—	—	—	2
e	26 (1)	10 (1)	—	—	—	—	—	3
(2) *a*	305 (3)	238 (4)	—	—	—	—	—	2
b	47 (1)	46 (2)	—	—	—	—	—	2
c	82 (2)	84 (6)	—	—	—	—	—	3
d	46 (2)	61 (6)	36 (10)	—	—	—	—	3
e	102 (4)	99 (8)	92 (16)	—	—	—	—	2
(3) *a*	66 (2)	44 (2)	27 (2)	17 (2)	11 (2)	4 (2)	4 (2)	$\sqrt[5]{10}$
b	178 (2)	63 (2)	—	6 (2)	0 (2)	—	—	$\sqrt{10}$
c	180 (4)	27 (2)	6 (2)	2 (2)	—	—	—	$\sqrt{10}$
(4) *a*	264 (2)	25 (2)	—	—	—	—	—	10
b	476 (2)	39 (2)	—	—	—	—	—	10

Table 3. *Analysis of plaque counts from Table 2*

Experiment	$\hat{h}$	$\hat{I}$	u ($h = 1$)	u ($h = 1{\cdot}0164$)
(1) *a*	0·9664	48·31	−0·234	−0·348
b	1·0477	71·03	0·402	0·264
c	1·1232	21·50	0·571	0·495
d	1·2372	27·68	1·248	1·162
e	0·8697	8·72	−0·385	−0·433
(2) *a*	0·7729	64·23	−1·820	−1·951
b	1·0310	11·17	0·104	0·049
c	0·9781	50·08	−0·155	−0·271
d	0·8390	102·74	−1·632	−1·798
e	1·0739	93·73	0·715	0·557
(3) *a*	1·0435	89·11	0·411	0·256
b	1·0083	144·68	0·100	−0·097
c	1·1113	81·20	1·003	0·855
(4) *a*	1·0237	121·08	0·261	0·080
b	1·0865	191·12	1·196	0·969

It is still possible that the combined data from all fifteen experiments might contradict the one-particle model, and hence further analysis is desirable. The values from columns 2 and 3 of Table 3 may be substituted into (6) to give the weighted average $\bar{h} = 1{\cdot}0164$, with variance $(1126{\cdot}35)^{-1}$. The values $u_1, u_2, \ldots, u_{15}$, computed from (5) with $h = 1{\cdot}0164$, are given in the last column of Table 3, and their sum of squares is 11·15. The 50 % point of a chi-square distribution with 14 degrees of freedom is 13·34, so that the value obtained is not an unusually large one. Hence there is no evidence of heterogeneity among the fifteen experiments.

If h is set equal to the theoretical value 1, then (7) gives $z = 0{\cdot}550$. From tables

of the standardized normal distribution, the chance of a more extreme value is greater than 50 %. Hence the one-particle model is in accord with the combined data from all fifteen experiments.

The preceding example shows that a statistical analysis applied to diverse experiments performed over a wide period of time and in different places can exhibit a convincing compatibility with the hypothesis in question ($h = 1$). The results of such an analysis would seem to be more compelling than solely examining point estimates $\hat{h}$ or graphs of plaque count *vs.* dose.

We should like to thank Dr W. S. Rickert and Dr W. F. Forbes for helpful suggestions.

APPENDIX

Computation of $\hat{h}$ for a single series

Let y_i and y_j be the two largest plaque totals, and define

$$\tilde{h} = (\log [n_i y_j / n_j y_i]) / (i - j) \log d.$$

If the series has only two dilution levels ($k = 1$), then $\hat{h} = \tilde{h}$. However if $k > 1$, $\tilde{h}$ gives only a first approximation to $\hat{h}$, and additional calculations are required. Let h_1 denote some initial guess at the value $\hat{h}$. (For instance, one might choose the proposed theoretical value, or else take $h_1 = \tilde{h}$). Putting $h = h_1$, one computes θ, B, A, D, S, and I using the formulas (1) to (4). A closer approximation to $\hat{h}$ will then be given by $h_2 = h_1 + \Delta$, where $\Delta = S/I$. This procedure may now be repeated with h_2 as the new initial value to obtain yet a better approximation, $h_3 = h_2 + \Delta$. One continues in this fashion until the correction factor Δ becomes sufficiently small.

For example, consider the data of isolate one in Table 1. Here

$$d = \sqrt{10}, \quad k = 2 = n_0 = n_1 = n_2, \quad X = 134, \quad \text{and} \quad T = 14.$$

For an initial guess at $\hat{h}$ one may select the theoretical value, $h_1 = 2$, and the following results are then obtained:

$$\theta = 0{\cdot}1 \qquad B = 2{\cdot}22 \qquad A = 0{\cdot}24 \qquad D = 0{\cdot}28$$

$$S = 0{\cdot}5601 \qquad I = 20{\cdot}23 \qquad \Delta = 0{\cdot}02755$$

Hence a closer approximation to $\hat{h}$ is

$$h_2 = h_1 + \Delta = 2{\cdot}02755.$$

The calculations are now repeated using $h = 2{\cdot}02755$ to give

$$\theta = 0{\cdot}096878, \quad B = 2{\cdot}21253, \quad A = 0{\cdot}231297 \quad D = 0{\cdot}268839$$

$$S = 0{\cdot}009563, \quad I = 19{\cdot}64, \quad \Delta = 0{\cdot}00049$$

An even better approximation to $\hat{h}$ is then given by

$$h_3 = h_2 + \Delta = 2{\cdot}02804,$$

which is correct to at least three decimal places.

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J. Hyg., Camb. (1974), **73**, 35
Printed in Great Britain

A possible relation between human pathogenicity of smallpox vaccines and virus growth at elevated temperatures

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(*Received* 31 *October* 1973)

SUMMARY

Seven smallpox vaccines of known human pathogenicity were tested for their ability to produce pocks on the chick chorioallantois at 39·7° C. Significant differences were found and the more pathogenic strains produced pocks with greater efficiency at 39·7° C. than did strains of average or low pathogenicity.

INTRODUCTION

Clinical trials have shown differences in the human pathogenicity of smallpox vaccine strains. For instance Polak *et al.* (1963) showed that the order of human pathogenicity of 4 vaccines was Lister < Ecuador < Bern < Copenhagen. More recent trials have shown the attenuated strain, CV-1 to be less pathogenic than the Lister and Wyeth strains (Kempe, 1968; Ducksbury *et al.* 1972; J. G. Galasso, I. Tagaya, personal communication).

Little progress has been made in the search for simple laboratory markers which correlate with human pathogenicity. Bektemirov, Shenkman & Marennikova (1971) showed a correlation between interferon resistance and pathogenicity for mice and rats. Various studies, however, have shown that when strains are listed in their order of pathogenicity for laboratory animals, the order determined is not the same as the order of pathogenicity for man (e.g. Turner, 1967; Anderson, 1969; John, 1969; S. S. Marennikova, personal communication).

The present paper suggests a possible relation between the human pathogenicity of smallpox vaccine strains and their ability to produce pocks at elevated temperatures on the chick chorioallantoic membrane (CAM).

MATERIALS AND METHODS

Virus strains

In all 7 vaccines were tested, the Lister, Copenhagen, Bern, Ecuador, CV-1, Tashkent and Wyeth strains.

Quantitative ceiling temperatures

After inoculation onto the CAM, groups of fertile chick embryos were held at either 35° C. or at various experimental temperatures in special incubators with constant recording thermometers and thermostats accurate to $\pm 0{\cdot}1°$ C. (Baxby

Table 1. *Relationship between pock production and human pathogenicity of smallpox vaccines*

Vaccine	% pock suppression at 39·7° C (± S.D.)	Relative human pathogenicity		
		'Index of* Pathogenicity'	Fever† 38·3° C (%)	Malaise‡ (%)
Copenhagen	13·5 ± 4·1	100	—	—
Tashkent§	26 ± 5·4	—	—	—
Bern	30 ± 5·1	94	—	—
Ecuador	50 ± 7·7	57	—	—
Wyeth	59 ± 8·1	—	45	—
Lister	63 ± 8·4	37	—	80
CV-1	76 ± 9·2	—	12	32

* From Polak *et al.* (1963). Figure given is days with fever > 38·9° C. Copenhagen = 100 %, others adjusted accordingly.

† From Kempe (1968). Figure is % vaccinees with fever > 38·3° C.

‡ From Ducksbury *et al.* (1972). Figure is % vaccinees with 'malaise' ('Fretfulness, irritability, anorexia and restlessness').

§ 'Highly pathogenic' (Marennikova *et al.* (1969)).

1969). Pocks were counted after 48 hr. and the degree to which pock production was decreased at the experimental temperature was assessed.

RESULTS

As a result of preliminary experiments 39·7° C. was selected as the test temperature. With higher temperatures chick embryo deaths increased (Bedson & Dumbell, 1961) and the pocks produced by some strains, notably Wyeth, CV-1 and Copenhagen strains, changed to a very flat grey type which was sometimes difficult to count; the problems of changes in pock character at different temperatures have been discussed elsewhere (Baxby, 1969).

The results obtained at 39·7° C. are shown in Table 1, the vaccine strains being placed in order of efficiency of pock production at that temperature. It can also be seen that the same order is maintained when the strains are listed in order of human pathogenicity. The differences obtained in pock reduction tests with different strains were not great but, with attention to inoculation technique and temperature control, were very reproducible. Pock production by the least pathogenic strain CV-1 was reduced by about 75 %, that by the most commonly used strains, Wyeth and Lister, by about 60 %, whilst that by the most pathogenic strains was reduced by 25–30 % for Tashkent and Bern, and by 13 % for Copenhagen.

The data on human pathogenicity are drawn principally from the extensive trial of Polak *et al.* (1963) together with more limited trials which have compared CV-1 with either Wyeth or Lister vaccines. An extensive American trial, still in progress, should also provide valuable information. The results so far indicate the attenuated nature of the CV-1 strain and show the Wyeth and Lister vaccines to be similar to each other, the exact values for morbidity being dependent on titre of vaccine and route of inoculation (J. G. Galasso, personal communication).

DISCUSSION

The results presented here suggest a possible relation between the human pathogenicity of smallpox vaccines and growth on the chick chorioallantois at elevated temperatures. It is of interest that a similar relation is suggested by the work of Nizamuddin & Dumbell (1961) and Bedson, Dumbell & Thomas (1963) on different strains of smallpox virus.

A suitable smallpox vaccine must of course offer considerable protection against smallpox. This is usually estimated by serological studies and/or revaccination. Although early work with CV-1 suggested a high rate of seroconversion (Kempe, 1968) more recent studies suggest that seroconversion and resistance to revaccination is lower than it is with Lister and Wyeth vaccines (Ducksbury *et al.* 1972; J. G. Galasso, I. Tagaya, personal communication).

Despite the success of the WHO smallpox eradication campaign it is possible that smallpox vaccine development will continue. The selection of strains which show reduced pock production at elevated temperatures should provide a convenient initial stage in the development of further vaccines.

I would like to thank Drs H. G. S. Murray, R. Gispen, J. Leerhoy, A. J. Lee and G. Appleyard for providing virus strains, and Drs J. G. Galasso, H. Tint, M. F. Polak, K. McIntosh and I. Tagaya for providing information in advance of publication.

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J. Hyg., Camb. (1974), **73**, 39
Printed in Great Britain

Laboratory tests of 5-*p*-chlorophenyl silatrane as a rodenticide*

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SUMMARY

The properties of 5-*p*-chlorophenyl silatrane as a rodenticide against *Rattus norvegicus* and *Mus musculus* were investigated in the laboratory. The high oral toxicity of the compound was confirmed. When the compound was given to laboratory rats and mice by stomach tube at lethal dosages, signs of poisoning were observed within a minute. When caged wild rats and mice were given a choice between plain and poisoned baits the optimum rodenticidal concentration in the bait was about 0·5% for both species, producing 50% mortality in wild rats and 95% mortality in wild mice. The results are discussed in relation to safety in use and the probable effectiveness of the compound as a rodenticide in field conditions.

INTRODUCTION

In the United Kingdom the problems encountered in rodent control with the appearance and spread of resistance to the widely-used anticoagulant rodenticides have given increased emphasis to the need for new rodenticides that are both safe and effective in use (Greaves, 1971). The compound 1-(*p*-chlorophenyl) 2,8,9-trioxa-5-aza-1-silabicyclo (3,3,3) undecane, commonly known as 5-*p*-chlorophenyl silatrane, has been developed as a quick-acting rodenticide by M & T Chemicals Inc., and proposed for use against several rodent species in the United States. Two substantial assets have been claimed for the compound from the point of view of safety in use. First, though it has high acute oral toxicity, its percutaneous toxicity is very low. Second, the compound hydrolyses to non-toxic products after a short time in the presence of moisture, in prepared baits and in the bodies of poisoned rodents, which minimizes the hazard of persistent residues to wild-life and domestic animals (Beiter, Schwarcz & Crabtree, 1970).

This report describes laboratory trials carried out with 5-*p*-chlorophenyl silatrane on the Norway rat (*Rattus norvegicus*) and the house mouse (*Mus musculus*). The main aim of the study was to determine suitable bait concentrations of the compound for use in the field trials described in the two accompanying papers (Rennison, 1974; Rowe, Swinney & Bradfield, 1974).

METHODS

The animals employed included male laboratory rats (Wistar) and mice (LAC Grey) and wild rats and mice of both sexes. The wild mice were laboratory-bred, first or second generation descendants of wild-caught mice. The wild rats included warfarin-susceptible animals, caught in a Midlands refuse destructor and held in the laboratory for at least three weeks before use, and also warfarin-resistant rats caught on farms in the Welshpool area that had survived for at least two weeks in the laboratory after a single subcutaneous dose of 200 mg/kg of warfarin.

Doses of 5-*p*-chlorophenyl silatrane were given by stomach tube to the laboratory animals grouped five to a cage. The compound was ground finely in a mortar, suspended in a 5 % solution of powdered acacia B.P. and then administered to the animals within 15 min. A watch was kept on the treated animals and the time of onset of signs of poisoning was recorded.

Feeding experiments were carried out with singly-caged wild rodents. Each animal was presented with a choice for 48 hr. between plain and poisoned, but otherwise identical, baits. The bait base consisted of 90 % pinhead oatmeal, 5 % wholemeal wheat flour and 5 % corn oil. The two baits, mixed freshly each day, were provided in similar amounts and in sufficient quantity to permit each animal to feed exclusively on the unpoisoned bait without going hungry. The positions of the two baits in each cage were interchanged after the first 24 hr. in order to minimize any effects that place preference might have had on bait consumption. Mortality and the amounts of bait eaten were recorded daily. Animals that died were autopsied and survivors were kept under observation for 7 days after their last exposure to the poison.

We are indebted to M & T Chemicals Inc., Rahway, New Jersey, U.S.A. for providing a supply of 5-*p*-chlorophenyl silatrane. A sample of the compound was returned to the supplier midway through the work and was found to have suffered no significant decomposition in storage.

RESULTS AND DISCUSSION

Oral intubation tests

The results of oral intubation tests with laboratory animals are given in Table 1. The mortality data are consistent with the acute oral LD 50 estimates given by Beiter *et al.* (1970) of 1–4 mg/kg for rats and 0·9–2·0 mg/kg for mice. After a single dose of 10 mg/kg all animals had convulsions within 1 min. and died within 5 min. There was no evidence of subacute toxicity in the animals given successive daily doses and the two rats that died did so immediately after the second dose was administered. No symptoms of illness were seen in any of the animals that survived and at autopsy there were no obvious signs attributable to the poison.

The short interval between dosing and the onset of illness may be compared with corresponding estimates obtained by various techniques of 15 min. for norbormide (Greaves, 1966), 47 min. for sodium fluoroacetate and zinc phosphide and 157 min. for fluoroacetamide (Bentley & Greaves, 1960). These intervals provide a measure of the time available to a rodent for the continued ingestion of

Table 1. *Results of oral intubation tests on male laboratory rats and mice with* 5-p-*chlorophenyl silatrane*

Animals	Mean body weight (g)	Single dose		Four daily doses	
		10 mg/kg	1 mg/kg	1 mg/kg	0·1 mg/kg
Rats	105	5/5	0/5	2/5	0/5
Mice	20	5/5	0/5	0/5	0/5

Table 2. *Results of giving wild rodents a choice for two days between plain bait and bait containing* 5-p-*chlorophenyl silatrane*

Type of animal	Mean body weight (g)	Sex	Conc (%)	Mortality	Mean bait intake (g)		Mean and range of doses (mg/kg) of active ingredient taken by animals that:			
					Poison	Plain	Died		Survived	
Rat (non-resistant)	284	M	1·0	4/10	0·5	9·7	15	4–28	17	3–30
	213	F	1·0	5/10	0·2	2·2	9	3–16	9	0–21
	254	M	0·5	4/10	0·6	14·6	13	9–20	12	6–19
	306	F	0·5	7/10	0·6	5·0	7	3–15	19	10–27
Rat (resistant)	162	M	1·0	5/10	0·2	3·1	14	0–42	16	6–33
	103	F	1·0	9/10	0·2	1·6	23	1–43	0	—
	238	M	0·5	5/10	0·3	6·0	6	0–17	8	0–23
	245	F	0·5	4/10	0·3	9·3	5	3–7	5	0–9
Mouse	17	M	0·5	9/10	0·06	0·1	22	0–71	0	—
	13	F	0·5	10/10	0·06	0·2	27	0–111	—	—
	18	M	0·25	8/10	0·07	0·4	9	0–50	16	13–19
	13	F	0·25	9/10	0·07	0·1	14	0–63	21	21
	14	M	0·1	6/10	0·3	1·4	18	7–25	22	8–35
	15	F	0·1	6/10	0·2	1·0	9	7–15	17	0–35

poisoned bait. Since, as the above figures show, the onset of illness is relatively early with 5-*p*-chlorophenyl silatrane the opportunity for a rodent to ingest a lethal dose is correspondingly less than with the other rodenticides mentioned. This disadvantage may however be countered if the rate of ingestion of the compound can be increased by increasing its concentration in the bait.

Feeding tests with wild rodents

Preliminary feeding experiments with laboratory strains had indicated that the lowest concentrations worth testing in bait were 0·5 % for rats and 0·1 % for mice. These concentrations were therefore the first to be tried with the wild strains. Successively higher concentrations were then tested with further groups of animals until no appreciable increase in mortality was obtained.

The results are summarized in Table 2. Several animals, particularly mice, died after eating undetectably small quantities of poisoned bait and often these animals also appeared to have eaten no plain bait. However, only two mice and four rats

Table 3. *Chi square analysis of mortality in wild rodents (data from Table 2)*

Species	Source of variation	χ^2	D.F.	P
Mouse	Concentration	8·124	2	0·01–0·02
	Sex	0·414	1	0·5–0·7
	Sex × concentration	0·212	2	0·8–0·9
	Total	8·750	5	0·1–0·2
Rat	Sex	2·464	1	0·1–0·2
	Concentration	0·452	1	0·5–0·7
	Resistance	0·452	1	0·5–0·7
	Sex × concentration	0·457	1	0·3–0·5
	Sex × resistance	0·055	1	0·8–0·9
	Resistance × concentration	2·469	1	0·1–0·2
	Sex × resistance × concentration	2·458	1	0·1–0·2
	Total	8·807	7	0·2–0·3

survived without eating measurable amounts of poisoned bait and, of these, only one mouse had failed to eat at least some of the plain bait. It should be noted that overnight increases in the moisture content of bait of the order of 0·1 g can occur, which may account for some of the apparent failures to eat. Thus the majority, and probably all of the surviving animals did in fact eat a sublethal dose of the rodenticide and then avoided eating a further, lethal dose either by feeding preferentially on the plain bait or by virtually refraining from eating at all for the remainder of the experiment. A previous study of the feeding behaviour of rats in similar experiments indicated that these responses characterized the development of a learned aversion towards the poison and bait material (Greaves, 1966). It seems likely therefore, that if sublethal feeding on the bait were to occur in field conditions, the rodents would tend as a result to avoid eating further amounts of bait containing 5-*p*-chlorophenyl silatrane.

A chi square analysis of the mortality data is given in Table 3. Mortality was not significantly influenced by sex in either species or by resistance to warfarin in the rat. In mice the effect of increasing the concentration of poison in the bait was significant. Kills of 12/20, 17/20 and 19/20 were obtained at concentrations of 0·1, 0·25 and 0·5 % respectively. Since the mortality of 19/20 produced in mice by 0·5 % 5-*p*-chlorophenyl silatrane could scarcely be bettered, it seems likely that this concentration would be near-optimal for the control of house mice in field conditions. In rats the increase in mortality (from 20/40 to 23/40) obtained by increasing the concentration from 0·5 to 1·0 % was insignificant, which is consistent with the conclusion of Beiter *et al.* (1970) that a bait concentration of about 0·5 % is also optimal for the control of this species.

The very obvious difference between the two species in the mortality that occured in tests with the compound at 0·5 % suggests that it is likely to be more effective for the control of mice than of rats. Inspection of Table 2 shows that at this concentration rats generally ate less of the active ingredient (in terms of mg/kg) than did mice. The difference may be attributable to a species difference in ability to taste or smell the compound, though there is no direct evidence of

this. It seems at least as likely that a greater acuity on the part of the rats in detecting the earliest symptoms of poisoning may have contributed to their more frequent survival.

There remains the question of how 5-*p*-chlorophenyl silatrane is likely to perform in field conditions in comparison with other quick-acting rodenticides. As far as the mouse is concerned no comparable data appear to have been published for other rodenticides. The high kill obtained in the feeding test at a concentration of 0·5 % suggests however that in the field the success of treatments against mice is less likely to be limited by any failing of the rodenticide at this concentration than by the general difficulty of attracting the mice away from their normal food supplies to eat rodenticidal baits. Regarding the rat, the relation between the results of laboratory tests of the type used here and those of field trials have been discussed by Rennison, Hammond & Jones (1968) in the context of studies with the rodenticides zinc phosphide and norbormide. These authors report that mean kills of 54 % (13/24) with zinc phosphide and 29 % (7/24) with norbormide in laboratory feeding experiments with wild rats were reflected in the superior performance of zinc phosphide in field trials. The mean kill of 54 % (43/80) obtained here with 0·5 and 1·0 % 5-*p*-chlorophenyl silatrane suggests therefore that the compound is likely to be about as effective as zinc phosphide against rats in the field.

From the point of view of safety in use, it has been mentioned that 5-*p*-chlorophenyl silatrane has advantages over other rodenticides from the standpoints of the low toxicity of bait residues, low secondary poisoning hazard and low percutaneous and subacute toxicity. Nevertheless, the acute toxicity of the compound to non-rodents appears to be at least as great as that of other rodenticides (Beiter *et al.* 1970). It should therefore be realized that the hazard due to accidental ingestion of fresh bait is likely to be no less with this than with most other rodenticides.

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J. Hyg., Camb. (1974), **73**, 45
Printed in Great Britain

Field trials of the rodenticide 5-*p*-chlorophenyl silatrane against wild rats (*Rattus norvegicus* Berk.)*

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SUMMARY

Rattus norvegicus infestations on six farmsteads were poisoned with 0·5% 5-*p*-chlorophenyl silatrane and those on another six with 2·5 % zinc phosphide. Both poisons were applied in pinhead oatmeal bait containing also 5 % corn oil, after pre-baiting. The result of each treatment was assessed by comparing the take of pre-bait with that of a census bait (wheat) laid after the poisoning.

The zinc phosphide treatments were generally more effective than those done with 5-*p*-chlorophenyl silatrane, but the latter were somewhat detrimentally affected by cautious baiting on the part of one of the operators.

The results are discussed and it is concluded that although they indicate that 0·5 % 5-*p*-chlorophenyl silatrane may have approached zinc phosphide in effectiveness under the conditions of the trial, it would in most circumstances be significantly less effective and possibly less safe to use than the latter, well-tried poison.

INTRODUCTION

Six trials of 5-*p*-chlorophenyl silatrane for the control of common rats (*Rattus norvegicus*) were carried out on infested farms in Montgomeryshire and Shropshire where resistance to anticoagulant rodenticides is common and where, as a result, field staff were available who were fairly experienced in the use of acute poisons. The results given below have been abstracted from data covering more extensive trials in which the properties of several other acute toxicants besides 5-*p*-chlorophenyl silatrane were investigated and were compared with those of 2·5 % zinc phosphide, probably the most effective acute rodenticide in common use in Britain.

METHODS

Following the work of Beiter, Schwarcz & Crabtree (1970) and of Greaves, Redfern & Tinworth (1974), the concentration of 5-*p*-chlorophenyl silatrane used in the six trials was 0·5 %. Six control treatments were carried out at the same time with 2·5 % zinc phosphide.

Only farmsteads that on inspection appeared to have moderately heavy rat infestations were chosen for treatment. Farms with obviously lightly or heavily infested buildings were excluded because in practice the tendency to underbait

and therefore not kill a proportion of the rats increases with infestation size, particularly when, as in this type of trial, a standardized procedure has to be followed. The poisons were allocated to the farms at random. The two operators who carried out the treatments and worked independently of each other, were also randomly assigned farms to treat so that each used each poison at three sites. The same bait was used for all the treatments to avoid introducing baits as a source of variation in the results.

Each infestation was pre-baited for 5 days with a weighed amount of pinhead oatmeal containing 5 % corn oil. The largest daily amount of bait consumed by rats on either the 4th or 5th day was used as an index of the initial size of the rat population. The same bait base, freshly prepared with poison included was laid on the day that the prebait was removed and left down for 14–24 hr. depending on the conditions on each farm. The shortening of the baiting period in some situations, in the interests of safety, is not thought to have directly affected the results, because rat activity on farms is habitually nocturnal. Eight days later the pre-baiting procedure was repeated using dry whole wheat. As far as possible the wheat baits were laid at different sites from those used during pre- and poison baiting. This time the largest daily take of the 4th and 5th days was taken as the index of the size of the surviving population.

RESULTS AND DISCUSSION

Experience has shown that dry wheat and pinhead oatmeal + 5 % corn oil are consumed by rats in about equal amounts in the type of infestations used in these trials. Thus it may be assumed for each treatment that the weights of pre-bait and census bait eaten are directly proportional to the numbers of rats present before and after poisoning. From each pair of readings the percentage success achieved can therefore be calculated and this is recorded in Table 1.

It can be seen that the treatments done with 2·5 % zinc phosphide were generally more successful than those done with 0·5 % 5-*p*-chlorophenyl silatrane. For example, of the six treatments that apparently gave less than 75 % control, five had been carried out with the latter. However, there are two reasons why the difference in apparent success found here cannot be attributed wholly to the relative effectiveness of the two poisons.

First, it chanced that somewhat larger infestations fell to be treated with 5-*p*-chlorophenyl silatrane than with zinc phosphide. Second and more significantly, it was revealed by analysis of the results (unpublished) of the complete set of trials, of which the above formed a part, that Operator *A* was less successful with the more acutely toxic baits than Operator *B*, probably because he was much more cautious in bait placement. Table 1 suggests that his caution was more marked in the case of 5-*p*-chlorophenyl silatrane than in that of zinc phosphide, possibly because the former poison acted on the target animals much more rapidly. This rapid effect was demonstrated on more than one occasion in the trials by the appearance of dead and dying rats near poison bait only a few minutes after it had been laid in the evening. Both the above effects would tend to favour zinc phosphide.

Table 1. *The maximum weights of pre-bait (pinhead oatmeal and 5 % corn oil) and post-poisoning census bait (dry whole wheat) eaten in one day by rats during trials of the rodenticides* 5-p-*chlorophenyl silatrane and zinc phosphide on twelve farms*

	Six treatments with 2·5 % zinc phosphide			Six treatments with 0·5 % 5-*p*-chlorophenyl silatrane		
Operators	Pre-bait eaten (g)	Census bait eaten (g)	Success* %	Pre-bait eaten (g)	Census bait eaten (g)	Success* %
A	1100	200	82	3300	1500	55
	700	350	50	3750	1850	51
	2550	300	88	1100	500	55
B	600	150	75	2400	660	73
	1320	240	82	940	440	53
	1380	0	100	2060	370	82
Total	7650	1240	84	13550	5320	61

* Percentage success = 100 (weight of prebait – weight of census bait)/weight of pre-bait.

The main reason why 5-*p*-chlorophenyl silatrane was less effective might be that this same rapid action resulted in a number of rats developing warning symptoms before they had eaten a lethal dose. This might arise as a result of temporary disturbance during the first stages of feeding. Such disturbance may have included the development of overt poisoning symptoms in neighbouring rats that had begun to feed somewhat earlier – an occurrence that is believed not to be of any importance with poisons such as zinc phosphide that take longer to act.

CONCLUSIONS

It had been suggested by Beiter *et al.* (1970) that because 5-*p*-chlorophenyl silatrane rapidly decomposes in the presence of moisture, its use as a rodenticide minimizes the hazards to other wildlife and domestic animals. Clearly this is so if bait residues and dead rodents are not carefully picked up after poisoning; but at the same time the compound's instability in water reduces its potential as a rodenticide by restricting its use to dry environments. More importantly perhaps, the compound cannot be used in damp baits such as soaked wheat, which are generally more attractive to rats and so in many situations more efficient vehicles for acute poisons than dry baits.

The results of this trial therefore indicate that 0·5 % 5-*p*-chlorophenyl silatrane will be less useful for rat control than 2·5 % zinc phosphide except when the risks of secondary poisoning to non-target species are high. In practice, such situations are uncommon and more often it is the danger of primary poisoning that prevents operators from poison baiting effectively.

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J. Hyg., Camb. (1974), **73**, 49
Printed in Great Britain

Field trials of the rodenticide 5-*p*-chlorophenyl silatrane against wild house mice (*Mus musculus* L.)*

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SUMMARY

The performance of the rodenticide 5-*p*-chlorophenyl silatrane at 0·5 % in a wholemeal flour/pinhead oatmeal/corn oil bait was compared with that of zinc phosphide at 3 % in the same base in poison treatments carried out against urban infestations of the house mouse (*Mus musculus* L.). Each poison treatment was conducted for 1 day and after 3 days' pre-baiting. The success of the treatments was assessed from census baitings conducted before and after treatment. Treatment success varied considerably with both poisons used but in general 5-*p*-chlorophenyl silatrane proved to be at least as effective as zinc phosphide, a commonly used acute rodenticide for the control of mice.

INTRODUCTION

In the laboratory, 5-*p*-chlorophenyl silatrane performed well in oral dosing experiments using *Mus musculus* of the LAC Grey strain and also in feeding tests on LAC Grey and wild mice when it was included in bait at a concentration of 0·5 % (Greaves, Redfern & Tinworth, 1974). The results of field trials carried out in urban areas against free-living mice using the poison at the same concentration are presented below and compared with the results of similar treatments done with 3·0 % zinc phosphide.

METHODS

In each treatment poison bait was applied for 1 day after 3 days of pre-baiting. In all, six treatments were conducted with each poison in a miscellany of premises including kitchens, offices and shop-stores chosen at random from those available. The pre-bait and carrier used for the poison was the same as that employed in the laboratory tests (i.e. wholemeal flour (5 %), corn oil (5 %) and pinhead oatmeal (to 100 %)) except for the addition of 0·05 % of the dye chlorazol sky blue.

The percentage success of each poison treatment was calculated from the total amount of plain bait (canary seed, *Phalaris canariensis*) eaten during the last 3 days of 4 day pre- and post-treatment censuses. The census baits were laid on small wooden trays distributed at close intervals throughout the infested area. The trays were laid at the same sites in the two censuses, but the pre-bait, placed

Table 1. *Results of pre-baiting and 1-day poison treatments against mice using 5-p-chlorophenyl silatrane and zinc phosphide*

Treatment		Pre-treatment census Consumption of canary seed (g) on day				Poison treatment: Consumption of pre-bait (g) on day			Poison treatment: No. of visits to poison baits on day 1	Post-treatment census Consumption of canary seed (g) on day				Estimated success
Type	Trial no.	1	2	3	4	1	2	3		1	2	3	4	(%)
0·5 %	1	7	15	16	11	9	7	6	1	0	0	0	0	100·0
5-*p*-chlorophenyl	2	11	24	36	50	34	37	37	6	0	3	3	3	91·8
silatrane	3	—	21	25	20	8	5	13	*	—	2	5	9	75·8
	4	72	107	145	151	85	120	141	8	30	31	38	37	73·7
	5	17	34	37	49	31	37	33	*	0	0	0	0	100·0
	6	6	8	13	13	16	20	16	2	6	13	10	7	11·8
3·0 %	7	0	6	14	15	5	14	17	2	7	11	18	20	0·0
zinc-phosphide	8	11	13	15	15	23	16	15	4	2	1	0	4	88·4
	9	0	2	4	3	3	8	15	3	0	0	0	2	77·8
	10	5	14	9	10	15	12	15	2	6	6	8	9	30·3
	11	2	14	24	25	4	8	11	2	12	15	13	13	34·9
	12	11	17	14	14	11	9	9	1	2	2	3	6	75·6
Totals		142	275	352	376	244	293	328		65	84	98	110	

* Undetectable.

Table 2. *Analysis of the data in Table* 1 *using the pre-treatment census takes for days* 2–4 *as the independent co-variates of initial infestation size to adjust the dependent post-treatment takes*

Source of variation	Degrees of freedom	Mean square	Variance ratio (F)	Significance (P)
Residual error in post-treatment census	10	1005·4	—	—
Regression of post- on pre-treatment census takes in the error term	1	6809·9	—	—
Deviations from the regression	9	360·4	—	—
Between (adjusted) poison means	1	882·0	2·4	> 0·05

in the same number of trays, was put down at different sites. Pre-baiting was begun 7 days after the end of the pre-treatment census and the post-treatment census was carried out 7 days after the end of the poison treatment.

RESULTS AND DISCUSSION

The results of the comparative pre-baiting and 1-day poisoning treatments with 5-*p*-chlorophenyl silatrane and zinc phosphide are shown in Table 1. The daily amounts of canary seed and of pre-bait eaten at the censuses and during the pre-baiting period are also given – together with the numbers of poison baits visited by mice and the estimated percentage kills.

Table 1 shows that variable control resulted from both the 5-*p*-chlorophenyl silatrane and zinc phosphide treatments and that complete control was achieved in only two of the twelve treatments (nos. 1 and 5) – each time with 5-*p*-chlorophenyl silatrane. The total control obtained, calculated from the total amount of pre-treatment and post-treatment census bait eaten in days 2, 3 and 4, was 79·2 % in the case of the six 5-*p*-chlorophenyl silatrane treatments and 42·5 % for the zinc phosphide treatments. When however an analysis of co-variance was applied to the data in order to eliminate any effect attributable to infestation size, no significant difference between the efficacy of the two poisons could be demonstrated (Table 2). Thus it may be concluded only that 5-*p*-chlorophenyl silatrane would be a reasonable alternative acute poison to zinc phosphide for use against mice.

Although no treatments were conducted in which 5-*p*-chlorophenyl silatrane bait was laid directly, Table 1 shows that the consumption of pre-treatment census bait increased considerably between days 1 and 2 and that a less pronounced upward trend in the consumption of pre-bait also occurred during the pre-baiting period. The data tend therefore to support the conclusion of Southern (1954) that a short period of pre-baiting is advisable when acute poisons are used against mice that are well supplied with other food.

In a wider context the results of the trials emphasize the difficulty of drawing

to poison baits entire populations of mice existing in diverse environments and often living on a variety of foods, and of then ensuring, even if they are so drawn, that each individual consumes a lethal dose of the poison.

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J. Hyg., Camb. (1974), **73**, 53
Printed in Great Britain

Introduction of a cross-infection rate in children's wards and its application to respiratory virus infections

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(*Received* 16 *November* 1973)

SUMMARY

Statistical methods are described in detail for the calculating and comparing of cross-infection rates. In addition the use of these rates has been extended to study the influence of age and of different virus types on susceptibility to cross-infection in children's wards.

INTRODUCTION

Bacterial cross-infection in hospital is no longer the serious hazard it was in the past but in recent years attention has been drawn to the risks of virus cross-infection, particularly with respiratory viruses in children's wards (Sterner 1972; Ditchburn, McQuillin, Gardner & Court, 1971). In order to assess the extent of cross-infection, taking into account the days at risk of those children who enter hospital free of the infection and the days of possible infection imposed by those children who have the infection, a 'cross-infection rate' was devised (Gardner *et al.* 1973). In this paper the statistical methods for calculating and comparing cross-infection rates are described in detail. The use of the rates has been extended to study the influence of age and of different virus types on susceptibility to cross-infection.

METHODS

The period of study

The survey was undertaken from 14 December 1971 to 30 April 1972 when influenza A and respiratory syncytial (R.S.) viruses were epidemic.

Definition of cross-infection

Virus cross-infection was considered to have taken place when a child acquired an infection after being in the ward longer than the accepted shortest incubation period for the virus. For R.S. virus this period is 5 days and for influenza A, 1 day.

The wards

The wards studied for evidence of cross-infection with influenza A or R.S. virus were divided, as well as possible, into two groups – those of open design and those made up mostly of cubicles.

Group A wards (numbered for identification 1, 2, 3 and 4) had open sections with cots or beds for children over a year, together with a variable number of single cubicles which were used mainly for infants under 12 months, although, when necessary, older children were admitted. Group B wards (numbered 5, 6, 7 and 8) contained mainly single cot cubicles. A full description of the wards has been given previously (Gardner *et al.* 1973).

Virology and clinical categories of respiratory infections

The clinical category of each respiratory infection (Gardner *et al.* 1960), the types of specimens, methods of collection and laboratory techniques have been described elsewhere (McQuillin & Gardner, 1968; Sturdy, McQuillin & Gardner 1969; Ditchburn *et al.* 1971). In a previous paper (Gardner *et al.* 1973) the clinical picture and age incidence of illnesses produced by cross-infection have been described in detail. Examples to illustrate the ways in which cross-infection occurred in the wards were also given.

Cross-infection rate

When a cross-infection rate is being studied it is necessary to take into account not only the number of cross-infections which occur but also the number of susceptible child days and the number of child days of primary infection in the ward. Four factors which might have some bearing on the rate were not taken into account in these calculations:

1. the possibility of tertiary cases (becoming infected by a secondary case who had himself acquired the infection in the ward from a primary case),
2. the length of time of virus excretion by each infected child,
3. the adult carriage of viruses,
4. possible cases which occur after discharge from hospital.

The assumption has been made that these factors were similar in both groups of wards.

The formula for the rate is:

$$\text{Cross-infection per million susceptible days per infective day} = \frac{\text{Number of cross-infections} \times 10^6}{(\text{Number at risk} \times \text{mean stay}) \times (\text{Number of infected} \times \text{their mean stay})}.$$

The standard error of a rate R_1 is estimated as $R_1/\sqrt{n_1}$, where n_1 is the number of cross-infections in the ward or group of wards.

A difference between two rates may be tested for significance as follows:

$$z = \frac{R_1 - R_2}{\sqrt{\left[R_1 R_2 \left(\frac{1}{n_1} + \frac{1}{n_2}\right)\right]}}$$

z is compared with the standard Normal deviate. For example, a z-value $> 1{\cdot}96$ or $< -1{\cdot}96$ indicates a statistically significant difference between the rates at the 5% level.

It will be noted that this cross-infection rate, which is entirely valid for internal comparisons in this study, is nevertheless dependent upon the duration of the study itself. We would like to thank a referee for the suggestion that an alternative formula for comparison of studies of different durations could be achieved by multiplying our cross-infection rate by the duration of the study (in days).

RESULTS

During the 4 months of the study period, 154 children were admitted to hospital with illness due to R.S. virus infection and 13 acquired the infection in hospital. Over the same period, 56 children were admitted with illness due to influenza A virus and 15 acquired the infection in hospital. Table 1 shows in detail the numerical information required and the method of calculating the cross-infection rate of R.S. virus in both groups of wards. The nine cross-infections in Group A (open design) wards gave a rate of 7·1 and the four in Group B (cubicle) wards a rate of 4·2 cross-infections per million susceptible days per infective day.

These two rates were compared as follows:

$$z = \frac{7{\cdot}1 - 4{\cdot}2}{\sqrt{\left[7{\cdot}1 \,.\, 4{\cdot}2\left(\frac{1}{9} + \frac{1}{4}\right)\right]}} = 0{\cdot}884.$$

This value is less than the conventional 1·96 at $P = 0{\cdot}05$ and is thus not statistically significant.

For influenza A the 14 cross-infections in the Group A wards gave a rate of 31·0 cross-infections per million susceptible days per infective day, and in Group B wards one cross-infection gave a rate of 12·4. The difference between these two rates was not significant ($z = 0{\cdot}917$).

None of the individual wards experienced a large number of cross-infections and comparisons made between them were not statistically significant in either Group A or Group B for either type of infection.

When the ages of the children were considered some significant differences in the cross-infection rates emerged. For these comparisons the 'susceptible child days' were those for the particular age group but the infective figure was that for all ages. Table 2 shows in detail the method of calculating the cross-infection rates in three age groups, under 1 year, one year to 4 years, and 5 years and over.

Table 3 shows the numbers and rates of cross-infection for each age group in the two types of ward. From this, comparisons can be made between the age groups for both R.S. virus infection and influenza A. Comparison may also be made between the two types of infection at each age group. The period and places of survey were the same for both infections, and they were both epidemic during this time.

Table 1. *Calculations for cross-infection rates of R.S. Virus for Group A (open design) wards and Group B (cubicle) wards*

					Under 1 year		1 – 4 years		≥ 5 years		Number of infective (days) (5) × (6) + (7) × (8) + (9) × (10)	(4) × (11)	Number of cross-infections	Cross-infection rate $\frac{(13) \times 10^6}{(12)}$
	Total number admitted	Number at risk	Mean stay of those at risk (days)	Number of susceptible days	Number admitted with R.S. virus infection	Mean stay (days)	Number admitted with R.S. virus infection	Mean stay (days)	Number admitted with R.S. virus infection	Mean stay (days)				
Col. no.	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
							Group A							
Ward 1	370	351	5·8	2,036	15	10·8	4	9·0	—	—	198	403,128	3	7·4
Ward 2	323	306	7·0	2,142	13	10·9	2	11·5	2	12·0	189	404,838	2	4·9
Ward 3	95	81	10·5	850·5	7	11·1	6	4·3	1	6·0	110	93,555	0	0
Ward 4	309	275	6·7	1,842·5	23	6·7	11	3·9	—	—	196	361,130	4	11·0
												1,262,651	9	

Cross-infection rate for all Group A wards $= \frac{9 \times 10^6}{1,262,651} = 7{\cdot}1$

							Group B							
Ward 5	124	93	15·0	1,395	31	6·74	—	—	—	—	209	291,555	2	6·9
Ward 6	125	116	9·0	1,044	9	6·8	—	—	—	—	61	63,684	1	15·7
Ward 7	288	273	6·0	1,638	12	12·0	2	12·0	—	—	168	275,184	0	0
Ward 8	224	208	7·0	1,456	15	14·1	1	8·0	—	—	219	318,864	1	3·1
												949,287	4	

Cross-infection rate for all Group B wards $= \frac{4 \times 10^6}{949,287} = 4{\cdot}2$

Table 2. *Calculations for cross-infection rates of R.S. virus for three age groups in Group A (open design) wards*

	Under 1 year				Under 1 year		1–4 years		⩾ 5 years		Number of infective (days) (5) × (6) + (7) × (8) + (9) × (10)	(4) × (11)	Number of cross-infections	Cross-infection rate (13) × 10^6 / (12)
	Total number admitted	Number at risk	Mean stay of those at risk (days)	Number of susceptible days	Number admitted with R.S. virus infection	Mean stay (days)	Number admitted with R.S. virus infection	Mean stay (days)	Number admitted with R.S. virus infection	Mean stay (days)				
Col. no.	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
Ward 1	50	35	5·8	203	15	10·8	4	9·0	—	—	198	40,194	2	49·8
Ward 2	42	29	7·0	203	13	10·9	2	11·5	2	12·0	189	38,367	2	52·1
Ward 3	18	11	10·5	116	7	11·1	6	4·3	1	6·0	110	12,760	0	0
Ward 4	102	79	6·7	529	23	6·7	11	3·9	—	—	196	103,684	2	19·3
												195,005	6	

Cross-infection rate for children under 1 year in Group A wards = $\frac{6 \times 10^6}{195{,}005} = 30{\cdot}8$

	1–4 years													
Ward 1	124	120	5·8	696	15	10·8	4	9·0	—	—	198	137,808	0	0
Ward 2	115	113	7·0	791	13	10·9	2	11·5	2	12·0	189	149,499	0	0
Ward 3	36	30	10·5	315	7	11·1	6	4·3	1	6·0	110	34,650	2	57·7
Ward 4	207	196	6·7	1313	23	6·7	11	3·9	—	—	196	257,348	0	0
												579,305	2	

Cross-infection rate for children aged 1 to 4 years in Group A wards = $\frac{2 \times 10^6}{579{,}305} = 3{\cdot}5$

	5+ years													
Ward 1	196	196	5·8	1137	15	10·8	4	9·0	—	—	198	225,126	1	4·4
Ward 2	166	164	7·0	1148	13	10·9	2	11·5	2	12·0	189	216,972	0	0
Ward 3	41	40	10·5	420	7	11·1	6	4·3	1	6·0	110	46,200	0	0
Ward 4	—	—	—	—	—	—	—	—	—	—	—	—	—	—
												488,298	1	

Cross-infection rate for children aged 5 years and over in Group A wards = $\frac{1 \times 10^6}{488{,}298} = 2{\cdot}0$

Table 3. *Numbers and rates of cross-infection for each age group in Group A and B wards*

Age	R.S. Virus		Influenza A	
	Number	Rate	Number	Rate
	Group A wards			
< 1 year	6	30·8	1	11·2
1 to 4 years	2	3·5	11	55·2
⩾ 5 years	1	2·0	2	12·4
All ages	9	7·1	14	31·0
	Group B wards			
< 1 year	4	8·3	1	23·8
1 to 4 years	0	0	0	0
⩾ 5 years	0	0	0	0
All ages	4	4·2	1	12·4

Group A wards

The difference between the R.S. virus cross-infection rates of 30·8 for children under one year and 3·5 for children aged one to 4 years in the open design wards is significant ($z = 3{\cdot}22$, $P < 0{\cdot}01$). Comparing the rate of 30·8 for children under a year with the rate of 2·0 for children aged 5 years and over gives $z = 3{\cdot}40$, which is significant at the 0·1 % level.

Comparing the age groups for influenza A in the open design wards the children aged one to 4 years had a cross-infection rate of 55·2 which was not significantly higher than the rate of 11·2 for children under a year ($z = 1{\cdot}69$), but was significantly higher than the rate of 12·4 for children aged 5 years and over ($z = 2{\cdot}13$, $P < 0{\cdot}05$).

A comparison between the R.S. virus cross-infection rates and those for influenza A in the open design wards shows that only for the children aged one to 4 years are they significantly different; 3·5 compared with 55·2 gives $z = 4{\cdot}84$, $P < 0{\cdot}001$.

Group B wards

No cross-infections occurred in children aged over a year and with only four R.S. virus and one influenza A cross-infection occurring in the children under 1 year, the numbers are too small to make valid comparisons using the above method. An exact test based on the binomial distribution shows that there is no significant difference between the two groups.

A comparison can be made between the cross-infection rates in the two types of wards for children under one year; the difference between the R.S. virus rate of 30·8 in the open wards and 8·3 in the cubicle wards was statistically significant ($z = 2{\cdot}2$, $P < 0{\cdot}05$). For influenza A the rate of 11·2 for children under one year in the open wards was not significantly different from the rate of 23·8 in the cubicle wards ($z = 0{\cdot}55$).

DISCUSSION

The calculation of the cross-infection rate is simple and, as we have suggested in a previous paper (Gardner *et al.* 1973), could be of value in monitoring an existing situation or measuring the effect of a new one. Comparisons between cross-infection frequency in different centres might be made. If studies of different lengths were being compared, then for each study the cross-infection rate, as defined in this paper, should be multiplied by the duration of the particular study.

When separate age groups were compared some significant differences in the cross-infection rates emerged. Children under a year, in the open design wards had a significantly higher rate of R.S. virus cross-infection than children under a year in the cubicle wards. Within the open design wards the rate of R.S. virus cross-infection was significantly lower among children over one year of age than among children under one year. This difference seems likely to be due to two factors. In the first place, illnesses due to R.S. virus infection are usually less severe in older children (Chanock *et al.* 1961), so that some children in this group may have acquired illnesses so mild that they escaped surveillance. Secondly, it may be that immune defences in older children reduce the quantity of virus antigen in the respiratory tract to levels which defy identification.

There is a contrasting pattern of cross-infection rates for influenza A. The rate for children aged 1–4 in Group A wards is the highest of the three age groups and, though not reaching statistical significance when compared with the rate for children under a year, is significantly higher than that for children of 5 years and over. It has been noted that children admitted to hospital with illnesses caused by influenza A infection are most commonly aged between 1 and 2 years (Brocklebank, Court, McQuillin & Gardner, 1972). This age distribution is in contrast to that for R.S. virus, which most often results in hospital admission during the first year of life (Public Health Laboratory Reports, 1972, 1973); and this is also the case, although to a lesser extent, for the other two most commonly identified respiratory viruses, parainfluenza virus types 1 and 3 (Downham, McQuillin & Gardner, 1973). No information is yet available about the age distribution of children with influenza A infection who are not admitted to hospital. However, the findings of this cross-infection study support the impression that children under the age of a year are in some way less susceptible to infection by this virus and are usually less severely ill than older children if they do become infected. Why the relationship between age and influenza A infection should differ from that for the other common respiratory viruses is a matter for speculation, but it may point to important contrasts in mechanisms of pathogenesis and immunity.

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J. Hyg., Camb. (1974), **73**, 61
With 3 plates
Printed in Great Britain

An evaluation of a partial-walled laminar-flow operating room

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(*Received* 23 *November* 1973)

SUMMARY

This paper contains an assessment of the physical performance of a permanently installed down-flow laminar-flow operating room at the London Hospital. This system employs partial walls extending 0·76 m (2·5 ft.) from the ceiling, from which the air is allowed to issue freely downwards at an initial velocity of about 0·4 m./sec. (80 ft./min.).

The usefulness of the partial wall, as compared with a free issuing system, was demonstrated and a comparison made with a fully walled system. It was shown that a fully walled system would be more efficient than a partial-walled system as there was a loss in air velocity of about 20–25% with the partial wall due to the nonconstrained flow of air. This loss would be reflected in an increase in airborne bacterial count and would mean that an increase of 20–25% in the air volume would be required to obtain the same conditions as with the full-walled system. Entrainment of contaminated air was demonstrated but it was concluded that this would be of little consequence in the centre of the clean area, i.e. at the wound site. Sterile instruments, etc., however, on the outside of the clean area, would be more liable to airborne contamination.

Bacterial and dust airborne counts taken during total hip operations gave a very low average figure (0·3 bacteria/ft.^{3} or 10·5/m.^{3}) from which we conclude that the system was about 30 times cleaner in terms of airborne bacteria than a well ventilated conventional operating-room. We concluded that although the partial-walled system was slightly less efficacious than a normal full-walled system, the freedom of movement and of communication for the operating team could in some circumstances outweigh this disadvantage.

Sound levels were such that normal conversation was possible with little or no awareness of background noise.

INTRODUCTION

Within the last few years there has been considerable interest shown by surgeons, mainly orthopaedic, in the development of ultra-clean operating-room environments. The stimulation of this interest may be ascribed to two

independent lines of thought – those of Professor John Charnley in this country (Charnley, 1964) and the workers of Sandia Corporation in the U.S.A. (Whitfield, 1962). Professor Charnley was concerned with preventing sepsis after total hip replacement operations, and the U.S.A. workers were interested initially in preventing dust particles contaminating electronic components to be used in the space programmes. These two lines of research have now become intermixed and developed with the ultimate aim of providing medical personnel with bacteria-free environments. However, as this field of work has developed only within the last decade much research and development has still to be carried out before ultra-clean operating-rooms can be satisfactorily designed to as high a building standard as is shown in modern conventional operating-rooms.

A conventional ventilated operating-room is reasonably effective bacteriologically, in that the bacterial count of the air is usually kept below 175 bacteria per $m.^3$ (5 bacteria per cu.ft.), but if cleaner conditions are required it will be necessary to adopt the method of ventilation usually described as 'laminar-flow' (Whyte, Shaw & Barnes, 1973). This system supplies sterile air through an end wall or ceiling in a unidirectional manner at velocities up to 0·5 m./sec. (100 ft./min.). The air is normally supplied through a filter bank on the wall or ceiling which is much smaller than the dimensions of the room, and in order to prevent mixing of this clean air with the room air, walls are provided to constrain the air supplied. This barrier however puts constraints on the surgical team and curtails their freedom of movement and communication. In order to overcome this problem the system at the London Hospital, an assessment of which is reported in this paper, was built initially as a 2·5 m. × 2·5 m. (8 ft. × 8 ft.) ceiling supply system with air being allowed to pass freely into the operating room in, it was hoped, a unidirectional manner. It was subsequently modified with the provision of partial walls which extended 0·76 m. (2·5 ft.) down from the ceiling.

This departure from the normal use of the full walls has obvious advantages and was, as far as we were aware, the first operating room employing laminar-flow methods with a non-constrained downflow of air. Research is, however, being carried out in Holland on a similar idea (Bossers, 1973) and there also exists the Allander system (Allander & Abel, 1968) which is produced commercially and is a low-velocity unidirectional system which supplies air downwards from an area above the table, the periphery of the supply area being provided with air curtains to assist the downward supply of sterile air. A cross-flow non-constrained system is also available commercially in the U.S.A. from Agnew-Higgins Ltd, and is widely used there. Criticism has been levelled at these types of systems because of their relative inability to prevent entrainment from outside the clean area of contaminants which could reach the critical areas. The method of air supply employed at the London Hospital therefore merited investigation. Our findings constitute a major part of this paper.

In our experience most present day laminar flow systems are noisy, because well over 95% of the systems installed in operating-rooms today are of the prefabricated type with the fans installed inside the system and within the operating room. There is usually insufficient room to install silencing. It is doubtful if pre-

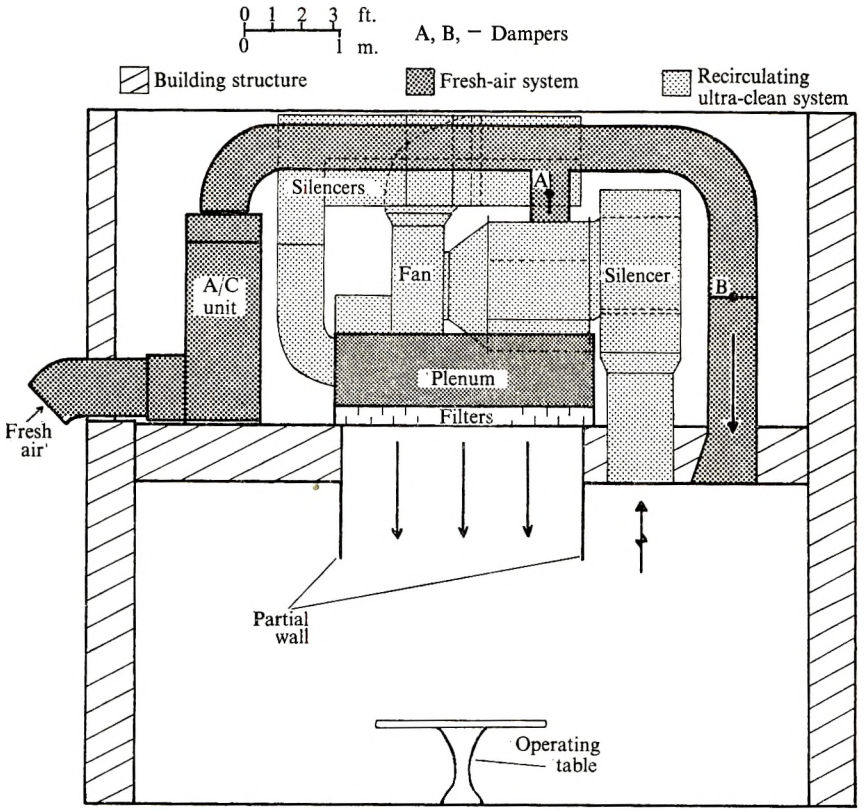

Fig. 1. Sectional elevation of operating-room at the London Hospital, with associated ventilation system dampers set at laminar flow position.

fabricated systems will ever be fully satisfactory as far as noise is concerned, and although they will continue to be installed into theatres which require upgrading, it is likely that the trend will be to install unidirectional-flow systems permanently.

The system in the London Hospital was the first to be permanently installed in this country, but it certainly will not be the last, and for this reason it was necessary to investigate its performance and report on any design problems which could be avoided in subsequent installations.

DESCRIPTION OF THE ULTRA-CLEAN OPERATING ROOM

Ventilation system

A sectional elevation of the operating room and its associated ventilation system is shown in Fig. 1. This shows the two alternative ventilation systems which may be selected in the operating room, one being a fresh air supply and the other a unidirectional air flow system. The two alternative systems were installed to permit direct comparisons to be made in the future between ventilation with ultra-clean air and conventionally filtered air in the same theatre.

When the full fresh air system is selected the air conditioning system draws

1·42 m.^{3}/sec. (3000 ft.^{3}/min.) of fresh air from outside, conditions the air and then ducts 0·85 m.^{3}/sec. (1800 ft.^{3}/min.) of this air to the operating room via two ceiling grilles. The remaining 0·57 m.^{3}/sec. (1200 ft.^{3}/min.) of this system is supplied to the anaesthetic room, which is adjacent to the operating room, through a ceiling grille.

When the unidirectional ultra-clean system is selected flap B closes the fresh air supply to the operating room and flap A is opened to allow the fresh air to mix with the recirculated air from the operating room. The ultra-clean recirculation system, draws approximately 2·20 m.^{3}/sec. (4700 ft.^{3}/min.) through the ceiling extract grille. This extract air is made up with 0·85 m.^{3}/sec. (1800 ft.^{3}/min.) of fresh air from the air conditioning unit which now follows the alternative route into the ultra-clean system. The total designed air volume supplied is therefore 3·05 m.^{3}/sec. (6500 ft.^{3}/min). which would give a velocity of 0·5 m./sec. (100 ft./min.) through the 2·5 m. × 2·5 m. (8 ft. × 8 ft.) filter ceiling. These design values were not reached however during this study. The excess air in the room (0·85 m.^{3}/sec. or 1800 ft.^{3}/min.) finds its way out of the operating room by flap dampers in the utility room and through cracks in the theatre doors, thus pressurising the operating area.

After being extracted from the theatre, the recirculated air is drawn through a set of silencers and into a large centrifugal fan. Once through the fan the air is ducted in twin systems to a plenum, again being passed through sets of silencers on the way. The air then passes through the H.E.P.A. filters mounted in the ceiling and hence into the operating-room via the constraints of the partial walls. Both the plants are controllable from the operating-room itself.

The pre-filter before the air conditioning plant and the secondary filters of the 100 % fresh air system are of the efficiency as recommended by the Department of Health and Social Security for fresh air, but the H.E.P.A. filters are not less than 99·97 % efficient against 0·5 μm. particles.

Internal construction

The internal construction of the operating room may be seen from Plate 1 which shows an actual operation in progress. The partial glass walls can be seen protruding approximately 0·76 m. (30 in.) into the room. Within this area it is possible to see the structural beams and twin light tracks which hold the twin Amsco operating-lamps. At the far end of the enclosure is a rectangular dark area which is the recirculated-air extract-grille. A drape can be seen hanging from the far end partial wall in order to prevent short circuiting of the supply air into the badly sited extract grille and to separate the anaesthetist and his equipment from the sterile area. One of the fresh air supply grilles is also shown on the extreme right-hand side of the ceiling. The operating team work within the vertical plane confines of the partial walls, the instrument trolleys being kept as tight round the working area as possible in order to keep the instruments in the sterile air flow.

RESULTS

Visualization of the air flow produced by the laminar-flow system

In order to study and report the flow of air at the London Hospital we adopted a method recently developed by the National Institute of Agricultural Engineering in Britain, and now described by Carpenter & Moulsley (1972). This system uses an air supply mixed with helium and a bubble generator to produce a flow of neutral-buoyancy detergent-bubbles of 3 mm average diameter. Plates 2 and 3 were achieved by photographing the bubble mass as a time exposure of 1 sec.

The light source was a series of spot lights set up in a duct, the light being projected through a slit facing the flow of air to be studied. The bubbles were illuminated and the background kept dark with a backcloth. Two bubble generators were used in our studies and it may be seen that the bubble movement is shown up as a streak, its length being proportional to the velocity of the streamline. Knowing the actual length of the bubble streak by reference to an object of known size in the photograph and the exposure time of the camera, the velocity of the air stream may be ascertained.

Plate 2A shows a typical photograph that was obtained by releasing bubbles outside the unidirectional air-flow area and observing the number that penetrated into the area which it was intended to keep free of contamination. It should be noted that the partial walls were not in position but notwithstanding this, the air movement was such as to prevent any great number of bubbles passing into the clean zone. Plate 2B shows a typical photograph obtained by releasing bubbles outside the clean area when the partial walls are in place. It may be observed that once again there is little entrainment of the outside air. Although slight differences may be observed in these two photographs, no conclusions should be made as there was as much variation within a set of photographs taken with or without the partial wall as between these two sets of photographs.

Plate 3A shows the situation that existed at the point where the light tracks, which passed across the sterile air supply area, were attached to the ceiling. This situation only existed, however, when the partial walls were not present. The negative pressure created under the tracks caused contamination to pass into the sterile zone to a large extent. It is obvious that these tracks would be better sited outside the clean sterile air supply area.

Plate 3B shows clearly the bad positioning of the exhaust system which is adjacent to the unidirectional supply. It is very clear that this is extremely unsatisfactory and the exhausts should have been sited at ground level, preferably an exhaust grille on each wall.

Particle challenge tests

In order to supplement the observations made with the bubble generator technique, further tests were carried out on the ultra-clean system. These were done with and without the partial walls and carried out by releasing smoke on the outside of the system and measuring the concentration of smoke that was found to have penetrated into the clean area. Almost any type of smoke source could

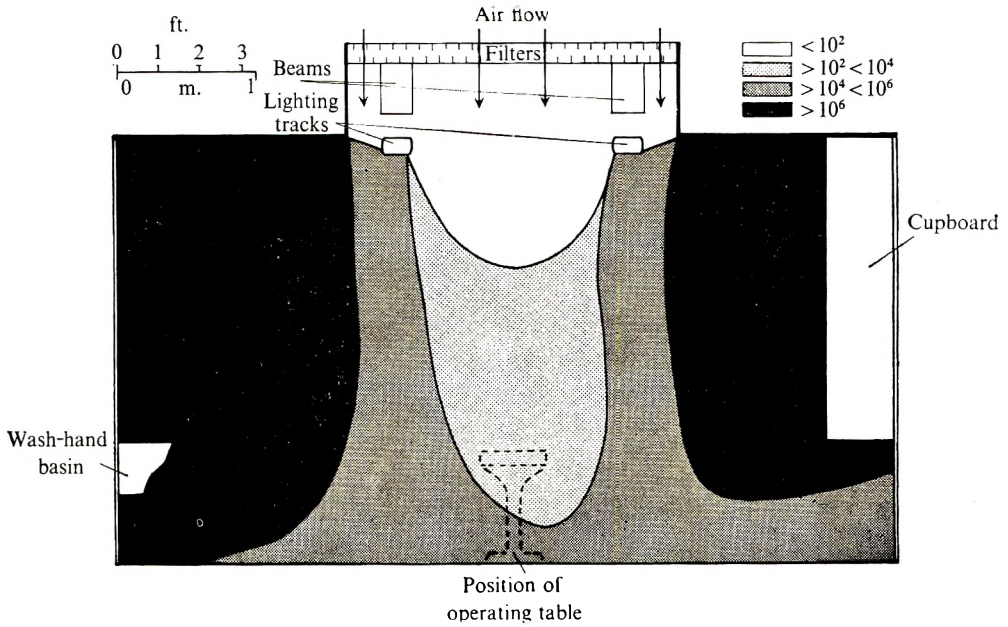

Fig. 2. Isopleth diagram of the non-constrained laminar-flow system.

have been used, but we have found that either joss sticks or cigarettes were simple and convenient. In this case we used joss sticks. The amount of smoke generated was adjusted by increasing or decreasing the number of burning joss sticks and by positioning them in order that the concentration outside the clean area of particles $\geqslant 0{\cdot}5$ μm. was just over 10^6 particles per ft.^{3} as measured by a Royco Particle Counter. In a given plane particle counts were taken and zones of equal contamination determined. These zones were $< 10^2$; between 10^2 and 10^4, between 10^4 and 10^6, and greater than 10^6 particles per ft.^{3} This type of diagram is used elsewhere, e.g. in the pollution research field where the lines of equal concentration are called isopleths. This name we have retained.

Fig. 2 shows an isopleth diagram obtained by studying the penetration of smoke particles at a plane half way across the supply face. The partial walls were removed for this test and the section studied in a plane such that the exhaust would not interfere with the air flow. The only thing therefore that prevented an ideal representation as to what should happen in a well constructed system was the interference of the light tracks, which not only drew in smoke along their length from outside the clean area (as shown in Plate 3A) but caused the air flow to curve inwards to the negative zone induced by them. This caused greater contamination than would have been achieved in the perfect situation.

Fig. 3 is an isopleth diagram of the system with the partial walls in place. These tests were carried out through the same plane as that used when the partial walls were not in place. The unusual feature in this diagram is the discovery that the air was being induced from outside the clean area, up the inside of the glass to a height level with the ceiling. The smoke was then blown down and across the clean area. This unfortunate effect was caused by a lower air velocity at that side of the

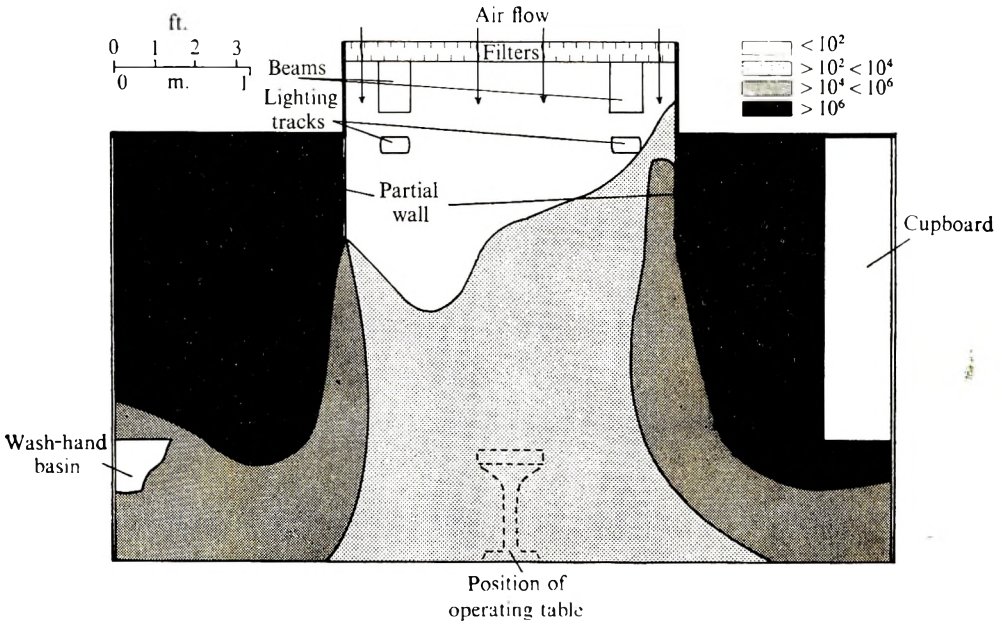

Fig. 3. Isopleth diagram of the partial-walled laminar-flow system.

supply area, so that the velocity was not great enough to overcome the effect of the areas of negative pressure produced by obstructions at the filter face. This would also appear to be the reason why this phenomenon was only exhibited with the partial walls in place, as the air velocity at the height of the glass from the floor was shown to be lower than that of ceiling height.

Although the positioning of the light tracks and the non-uniformity of the air velocity across the delivery area leads to disturbances of airflow which confuse assessment of the value of the partial walls, a larger clean area is produced at table height when the partial walls are used. Over an area approximately the same size as that of the air supply face (2·5 × 2·5 m.) the particle concentration was between 10^2 and 10^4 times less than that outside, the value at the wound site being near the lower of these ($1/10^4$).

Bacterial and particle concentrations in the operating room during several total hip operations.

Counts at the wound site. Samples of air were taken from the vicinity of the wound, rather than the theatre environment, as the latter may bear no relationship to the risk of airborne contamination. We therefore employed a method described previously (Whyte, Shaw & Barnes, 1973) and used a High-Volume Slit-Sampler (Casella Ltd., London) with an air flow of 700 l./min. (25 ft.^{3}/min.). This was connected by a bend to a long metal cone, which was sterilized between operations, and a sample was taken at a maximum of 15 cm. (6 in.) from the wound site. Because of the very large sample of air around the wound (25 ft.^{3}/min. or 700 l./min.) and the fact that the sampling point was half way along the incision and

Table 1. *Mean and standard deviation of bacterial concentrations at Killearn Hospital during total hip operations*

Velocity (m./sec.)	Mean (bact/m.^{3})	Standard deviation
0·2	34·8	34·8
0·3	10·2	7·3
0·4	2·8	1·04

below it, we felt that good sampling conditions were established. Bacterial samples were incubated for 36 hr. at 37° C. before counting.

Samples were also taken of dust particles of 0·5 μm. and over in diameter and of 5 μm. and over. This was done by means of a Royco Particle Counter, the sterile tube being clipped to the sampling cone and samples taken at the same spot as the bacterial sample. It was considered that as very few dust particles would pass through the high efficiency filters, any dust particles collected would normally be generated by potential sources of contamination, i.e. by the operating team.

Five total-hip replacement operations by three different operating teams were monitored. A total of thirty 10 min. airborne bacterial samples were taken, thereby sampling a total of 214 m.^{3} (7500 ft.^{3}) of air. The average bacterial count for each operation was 7·0, 14·0, 10·8, 13·3, 12·2 bacteria/m.^{3} (0·20, 0·40, 0·31, 0·38, 0·35 bacteria/ft.^{3}), the mean count of the 30 samples taken throughout the operations being 10·8/m.^{3} (0·31/ft.^{3}) with a standard deviation of 5·95 (0·17). One minute dust samples were taken throughout the period and gave an average dust concentration of 230,000/m.^{3} (6600/ft.^{3}) for particles $\geqslant 0{\cdot}5$ μm. and 13,000/m.^{3} (370/ft.^{3}) for particles $\geqslant 5{\cdot}0$ μm.

Given in Table 1 for comparison are the results obtained at Killearn Hospital, Scotland during a series of total-hip operations carried out in a completely enclosed down-flow laminar-air system (Whyte, Shaw & Barnes, 1973). Concentrations are given for downward velocities (measured 1 metre away from the filter face) of 0·2, 0·3, 0·4 m./sec. (40, 60 and 80 ft./min.).

Inspection of the results shows that at a down flow velocity of 0·3 m./sec. (60 ft./min.) the bacterial concentrations are very similar. The bacterial concentration was 10·8/m.^{3} (0·31/ft.^{3}) in the partial-walled system compared with 10·2/m.^{3} (0·29/ft.^{3}) in the full wall situation at Killearn Hospital. Inspection of the mean and standard deviation of the bacterial counts and a statistical comparison of the results (t–test) shows that the results obtained at the London Hospital are significantly different from those at Killearn Hospital at velocities of 0·4 m./sec. ($P \leqslant 0.01$) and 0·2 m./sec. ($P \leqslant 0{\cdot}05$).

Thus for a bacterial concentration of 10·8/m.^{3} the average concentration found at the London Hospital, it would be expected from our results at Killearn that the air velocity in the London Hospital system would be about 0·29 m./sec. This figure agrees well with our observations of air velocity as the measured velocity in the partial walled operating-room at operating table height was 0·23 m./sec. This figure was measured without a curtain present to screen the exhaust, and

therefore a slightly higher velocity could be expected when the curtain was present. This would bring the measured velocity closer to the predicted value.

Although no observations were made of these surgical teams operating in a conventionally plenum-ventilated operating-theatre it has been our experience in taking bacterial counts that average concentrations in the order of 315 bacteria/m^3. (9 bacteria/ft.^{3}) are associated with total hip operations. This would make the London Hospital's ultra-clean theatre about 30 times cleaner than modern conventionally ventilated operating-rooms.

It is interesting to consider the cleanliness of the air in terms of how many bacteria would be liable to be deposited from the air into an open wound. Using a simplified version of Stokes equation ($d^2 = Vg/0{\cdot}006$) the settling velocity (Vg) in ft./min. can be determined for particles of known equivalent diameter (d), where the equivalent diameter is the diameter of a sphere of unit density which has a settling rate in air equal to the particle in question. Noble *et al.* (1963) showed that the median diameter of bacteria-carrying particles in hospitals was about 13 μm. and using this figure it may be calculated that the number of particles being deposited on 1 ft.^{2}/min. would be equivalent to the number of particles in one cubic foot of air. This means that in the ultra-clean system a wound of area 1 ft.^{2} would have about 0·3 bacteria-carrying particles deposited per min. or 18 per hr.

Bacterial counts round the operating room periphery. It has been demonstrated in previous sections of this paper that a major consideration in assessing the capability of an ultra-clean system is its ability to prevent the ingress of contaminated material from the periphery of the operating room into the clean area. This ability has been assessed in previous sections but it is necessary to find out the dimension of the challenge, that is to say the amount of contamination that is present in the peripheral area. The less contamination that is present, the less important is the question of entrainment.

In order to carry out sampling in the area round the outside of the room the same type of bacterial sampler was used. This was placed on a trolley which was wheeled round the room in order to sample at various positions round the room. The sampling position was 2 m. (6 ft.) high. No particle counts were taken.

Twenty-five 2 min. air samples were taken during several operations. These samples varied from 11·2 to 315 bacteria per m.^{3} (0·32 to 8·98 bacteria per ft.^{3}) with a mean value of 70·7 per m.^{3} (2·02/ft.^{3}).

Velocity measurements

A comparison was necessary of the air velocity produced by a full-walled laminar-flow system and the partial-walled system from the same air supply. As we have demonstrated in previous experiments (Whyte, *et al.* 1973) that the efficiency of a laminar-flow system depends on its velocity it followed that if the velocity in the partial-walled system was considerably lower than in the full-walled system this would result in a significant drop in bacterial efficiency.

Measurements were made of the velocities from the filter face to the operating table height. These results are shown in Fig. 4. It may be seen from this figure that the velocity falls very quickly in the first few centimetres (Zone A) from

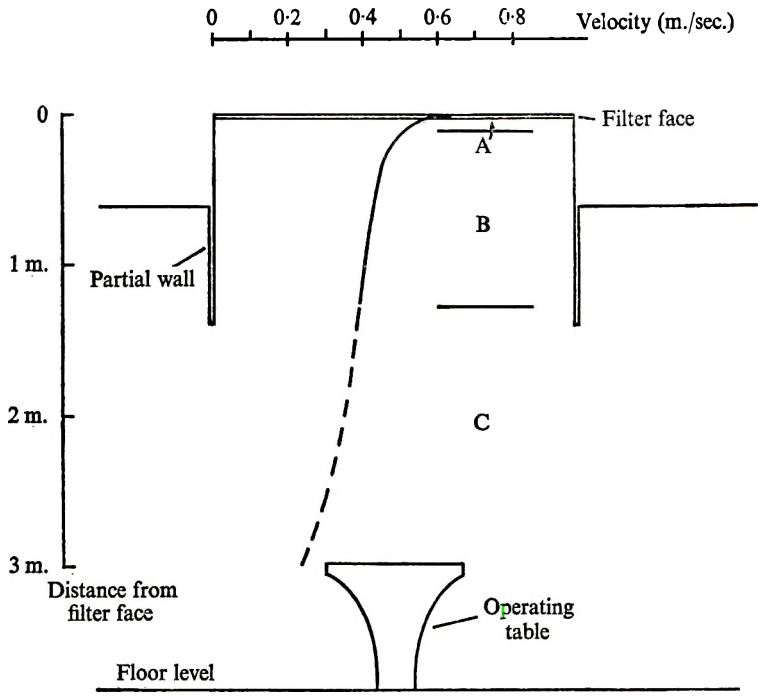

Fig. 4. Air velocity reduction from filter face to operating table.

0·63 m./sec. at the filter face to 0·50 m./sec. at 10 cm. distance. This initial drop may be attributed to jetting from the filter face producing high velocity areas which were picked up by our instruments.

After 10 cm. (Zone B) the velocity decreased gradually to a value of about 0·40 m./sec. at a plane level with the bottom of the partial wall, 1·25 m. from the filter face. This decrease in velocity of 20 % from the 10 cm. level to that of the partial wall is almost certainly caused by an increase in cross-sectional area of the opening. At the 10 cm. level there is a blockage area of approximately 20–25 % due to beams and main frames of the filters but once the air has passed these obstructions it opens out to the full area of the opening and hence the air speed drops. This velocity at the level of the bottom of the partial wall must be regarded as the *actual* air velocity the system is producing. Measurement of the air velocity at the filter face would be exaggerated both by the jetting of the air and failure to take into account the obstruction of the airstream. The supply velocity of the system should therefore be regarded as 0·4 m./sec. (80 ft./min.). This means that the volume of air supplied by the laminar-flow module was 2·5 m.^{3}/sec. (5100 ft.^{3}/min.) compared to the design figure of 3·05 m.^{3}/sec. (6500 ft.^{3}/min.).

On passing the bottom of the partial wall (Zone C) there is no constraint on the air and the air speed drops even further, reaching a value of approximately 0·23 m./sec. at operating table level, a reduction of 42·5 %.

These findings may be compared with those in a fully walled system. Velocity readings similar to the above series were taken in a down-flow laminar-flow system. We found that there was also a change in the velocity from a point 2 to 3 ft. from

the filter down to table height. The air velocity was found to drop from 0·4 m./sec. to 0·32 m./sec., a reduction of 20 %. This was caused by the fact that the air flow at table height was tending to veer away from the centre of the system in readiness to passing out at floor level round the periphery. It would appear therefore that the system with the partial wall is 20–25 % less efficient in terms of velocity achieved at table height than was the fully-walled system.

Sound measurement

It was expected that the sound levels found at the permanently installed London Hospital system would be much less than had been previously found in prefabricated systems built in this country, due to remoteness of the air-movement equipment and the opportunity for the inclusion of more silencing. This was the case at the London Hospital.

Measurements were taken in the empty room when the air was being supplied by the laminar-flow module and were an average of two readings taken on separate days. These results reflect accurately the noise generated by the laminar-flow system, as background noise was minimal. The operating room was close to a main trunk road but the fitting of double glazing had reduced the traffic noise to a very low level. The sound level for the theatre was N.C. 50 and except for a low-frequency rumble, possibly from the fan, at the 63 Hz. octave band, would have reached N.C. 45.

Comparative readings taken at the Princess Margaret Rose Hospital, Edinburgh in a prefabricated system gave an N.C. level of 65 whereas our own system at Killearn, which is also prefabricated, has an N.C. level of 60.

These readings are for a velocity as measured 1 metre from the filter face of about 0·45 m./sec. (90 ft./min.).

Subjective assessment by the surgeons showed the system not to be noticeably noisier than conventionally ventilated operating-rooms that they were used to and that normal conversation was possible with little or no awareness of background noise.

DISCUSSION AND CONCLUSION

It is our opinion that the partial-walled laminar-flow system at the London Hospital is a success. The success of the system may be considered in two ways – the access and convenience to the staff and its physical performance.

An obvious advantage of the partially walled system is that normal access is available at all times to the operating area and to the patient. Instruments, X-ray equipment, anaesthetic equipment and personnel can enter and leave the sterile area freely and in a conventional manner. The scrubbed team can also talk to other people in the theatre in a normal fashion; an important consideration especially in a teaching hospital.

From the engineering point of view several design faults existed which have in the main been overcome. However, as the laminar-flow room was the first to be permanently installed in the U.K., the building of the system could be considered

a research project and several lessons can be learned. These we should like to pinpoint in order that future designs may benefit from these mistakes.

Theoretically in terms of air cleanliness, a laminar-flow system without complete walls can not achieve the air cleanliness that one with walls can. However, we were interested to see how closely the partially walled system approached the fully walled system and whether or not it approached sufficiently to be acceptable. This question, the highlighting of the possible faults in the system, and the lessons to be learned were the main objects of our assessment.

Environmental conditions achieved and possible design faults

General design points. The height of the operating room ceiling was 10 ft. It was envisaged before installation that the air issuing from the 8 ft. square supply-area would be contaminated by some entrainment, but that at table height there would be a near particle-free zone where the operation could take place. After installation this was found not to be the case because (1) the distance from the air opening above to the required clean area below was excessive, (2) the filter face was obstructed by beams, (3) the light tracks which were fitted to these beams were so positioned as to fit flush with the ceiling and cause particles to be induced into the clean area and (4) the exhaust system was very badly sited adjacent to the air supply. All these factors came together to give a flow of air which was anything but unidirectional or particle free. Partial walls were therefore installed to overcome these effects. On testing the system with and without these walls we found that the walls produced substantial improvement; they completely prevented the contamination caused by the negative pressure induced under the light tracks and in part overcame some of the other faults.

No walls or partial walls? Because of the filter obstruction, light track and the exhaust, it was impossible to make an exact comparison of the flow of air with and without the partial walls. Theoretically it would seem that one would get a better air flow with less entrainment using the partial wall, but bubble generator and particle challenge indicated that in practice this was not a particularly obvious effect. However, apart from the possible theoretical advantage, the partial walls brought the sterile air supply closer to the critical area before the effect of entrainment and other forces could act on it. For this reason their use should be encouraged.

By challenging the laminar-flow system with smoke particles when the partial walls were fitted and then taken away, further evidence was obtained of the effectiveness of the system. The adverse effect of the lighting track and also the usefulness of the partial walls in extending the clean zone closer to the critical area were once again shown. We observed at one point a backflow of contamination up the inside of the partial walls towards the filters which reduced the effectiveness of the system. Conversation with staff at the London Hospital had led us to expect this phenomenon which was caused by obstruction of the air supply by beams and light tracks. It had been observed by smoke tests when the system was being first commissioned that this phenomenon was very noticeable and much greater than we had observed. However, in order to improve this the air velocity had been

increased and made more uniform across the filter face. This had been carried out before we tested the system and had obviously caused a great improvement.

Assessment of the partial-wall compared with a full-wall system. Smoke tests of the partial-walled system showed that in an area of approximately the same dimension as that of the air supply face (8 ft. × 8 ft.) the particle concentration was reduced to between 1/100 and 1/10,000 of the concentration that exists in the peripheral area of the operating room. This reduction was 100 times at the very outside of the clean area to 10,000 times in the centre. It is worth noting that the entrainment is less in this system than in a free flow jet as the barrier of the floor pushes the clean air out and hence the contamination is partially prevented from entering the clean area by this outwardly flowing air.

Bacterial sampling carried out in the area around the periphery of the ultra-clean system gave an average bacterial concentration of 70·7/m.^{3} (2·02/ft.^{3}) which would mean that entrainment of contamination in the partial-walled system would give an additional contribution of airborne contamination varying from 0·707 bacteria/m.^{3} (0·02/ft.^{3}) at the outside of the clean area to 0·00707/m.^{3} (0·002/ft.^{3}) in the centre.

Airborne bacterial sampling at the wound site during several total hip replacement operations shows that the average concentration would be in the order of 10·5 bacteria/m.^{3} (0·3/ft.^{3}) which would suggest that a negligible number of bacteria, less than 0·1 % of the bacteria in the area of the wound, were present as a result of entrainment. At the outer points of the 8 ft. × 8 ft. clean area, however, where the instruments could be lying exposed to the air, it would be much dirtier than a fully walled system, the majority of the contamination coming from the entrained air.

Although no bacterial sampling was carried out during total hip replacement in a conventionally ventilated plenum system we have no reason to suspect that it would differ from what we have normally observed, namely, an average concentration of 315 bacteria/m.^{3} (9/ft.^{3}). This would mean that the London Hospital system was 30 times more efficient than a good plenum ventilated operating-room.

It is interesting to note that, assuming a median diameter of 13 μm. for the bacterial particles in the air (Noble *et al.* 1963), the number of bacterial particles settling in the ultra-clean system into a wound of 1 ft.^{2} would be about 18 per hour.

The second factor that must be taken into account, apart from entrainment, is the effect of velocity. Because the air is not contained within full walls, the velocity at table height is between 0·80 and 0·75 of what could be achieved with walls. This is confirmed by airborne bacterial sampling where it was shown that the concentration at the wound site was what was expected from a velocity of 0·25 m./sec., the velocity at table level, rather than that from 0·40 m./sec., the velocity of the air issuing from the system. This means that the air volume of a partial-walled system must be increased by about 25 % to obtain similar conditions to those in a full-walled system.

Sound levels were achieved in this system which were much superior to the majority of prefabricated types of laminar flow systems which are installed

throughout the world and normal conversation was possible with little or no awareness of background noise.

This work was carried out under a Department of Health and Social Security contract but we also acknowledge the assistance of the Medical Research Council. We should also like to thank Mr Peter Bailey for his expert technical assistance.

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EXPLANATION OF PLATES

PLATE 1

Internal construction of the partial-walled operating room at the London Hospital.

PLATE 2

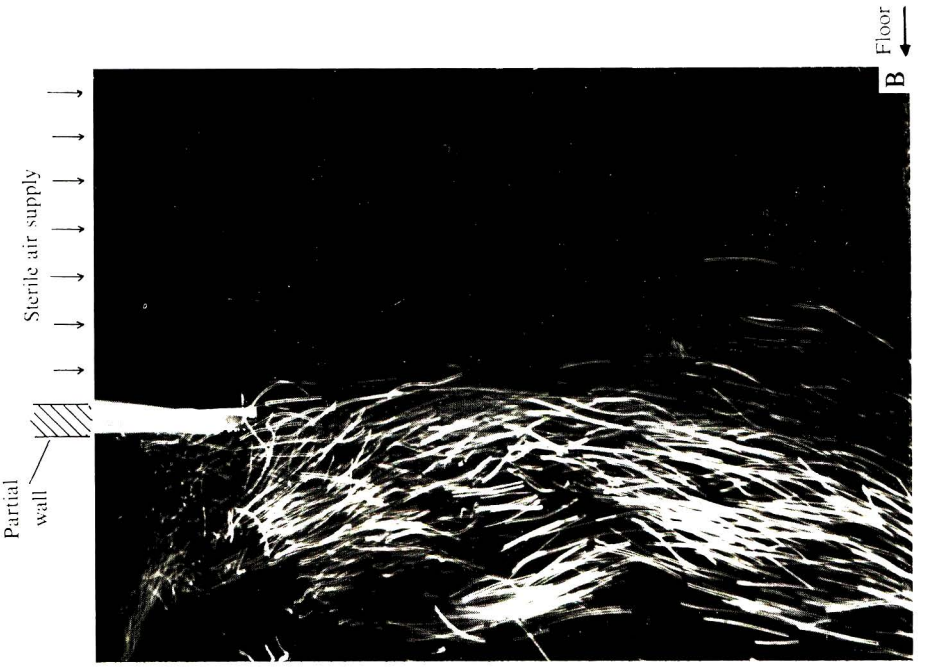
(A) Penetration of ultra-clean area. No partial wall.

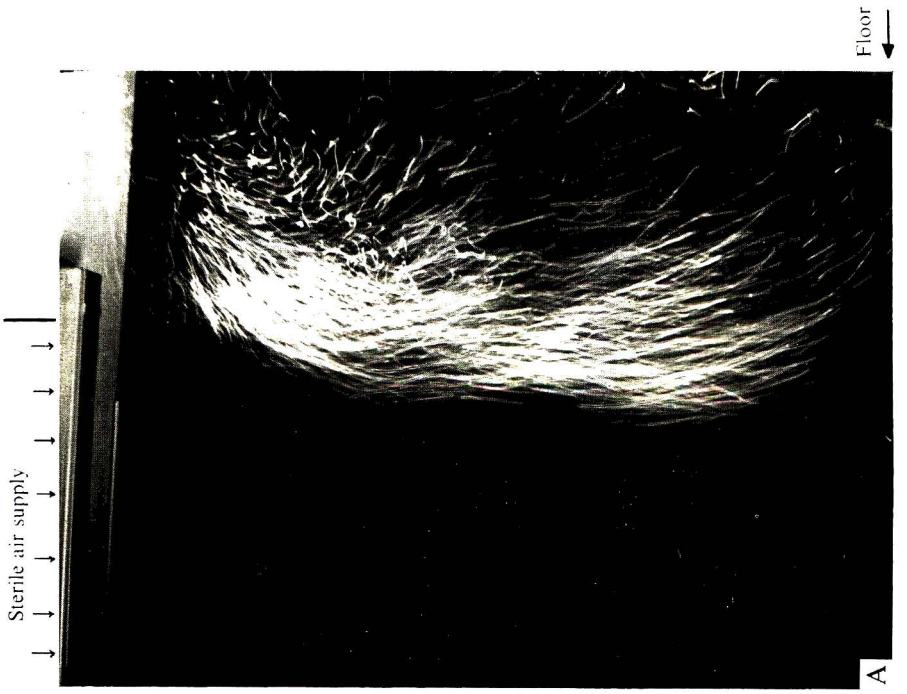
(B) Penetration of ultra-clean area. Partial walls in place.

PLATE 3

(A) The effect of light track straddling the air supply area.

(B) The effect of the adjacent extract system.


Floor
B
Sterile air supply
Partial wall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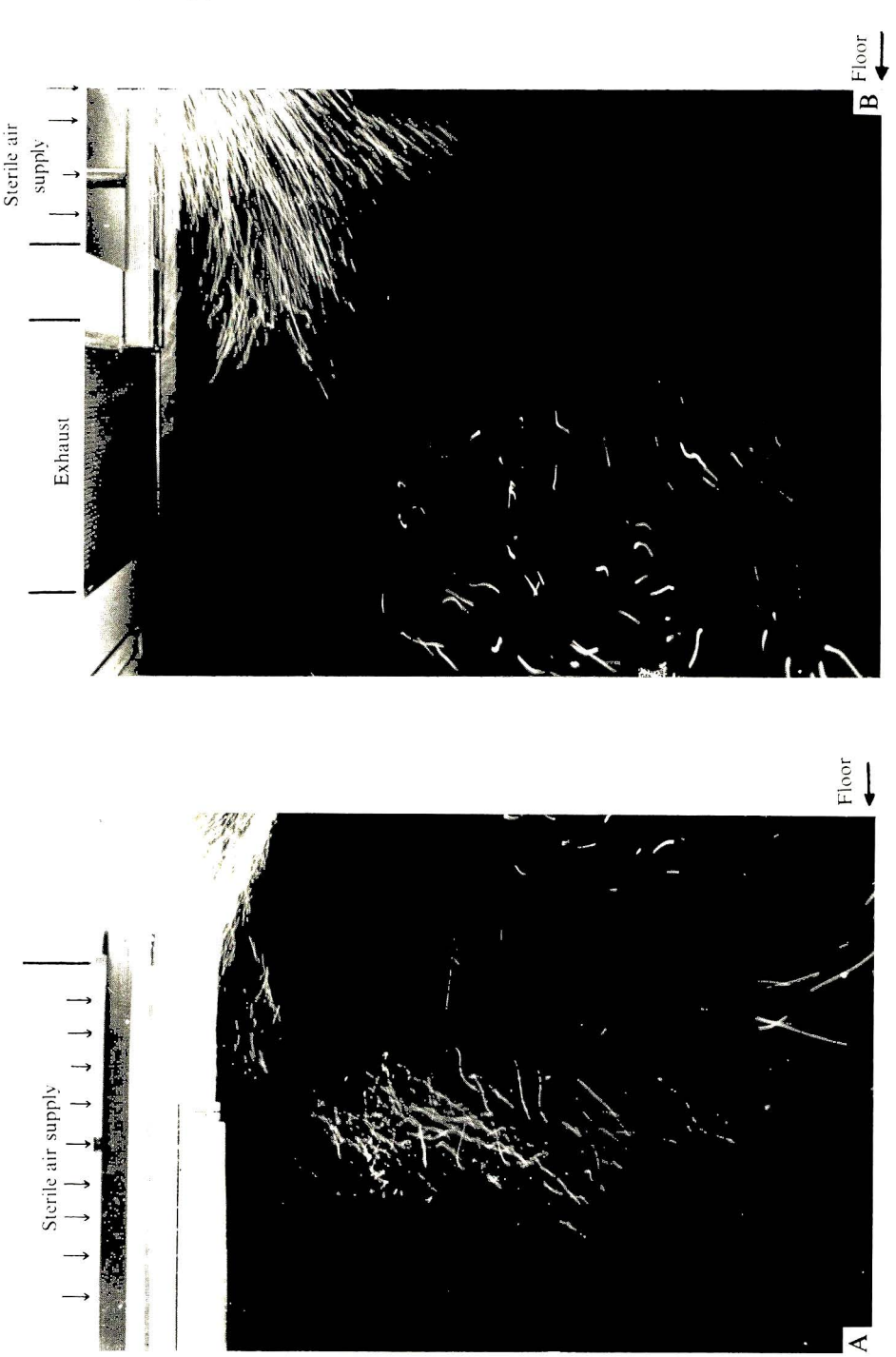
Sterile air supply
A


Sterile air supply
Exhaust
Floor
B
Sterile air supply
Floor
A

J. Hyg., Camb. (1974), **73**, 75
With 1 plate
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Specificity of the protective response induced by the slime layer of *Pseudomonas aeruginosa*

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(*Received* 29 *November* 1973)

SUMMARY

Active protection against *Pseudomonas aeruginosa* could be induced in mice by immunization with either the phenol killed cells or the alcohol precipitated fraction of the slime layer, or the ribosomal vaccine preparation. Passive protection could also be induced by injecting into mice antisera prepared in rabbits against these bacteria. This protection was due to the production of antibodies in reaction to the slime layer; the absorption of these antibodies by the slime caused the loss of protection. The fact that mice were also protected by vaccination with strains other than those used for challenging was attributed to the presence of an antigenically similar slime. Passive protection towards a heterologous strain, even one with an antigenically similar slime layer, was dependent on the dose of the challenging injection.

INTRODUCTION

In recent years, much attention has been given to the development of immunotherapeutic measures in order to supplement or replace the chemotherapeutic treatment of individuals who have been or could be infected with *Pseudomonas aeruginosa*. The need for such an approach arose because of the increased number of infections caused by this organism (Finland, 1970), especially following burns, and its resistance to the inhibitory action of antibacterial drugs. Recently progress has been made in preventing colonization of burns by the use of topical chemoprophylaxis. In this way the chance of invasive septicaemia has been reduced, but occasionally fatal infections have occurred even following this treatment (Lindberg *et al.* 1965).

Previous studies have shown that infection due to *Pseudomonas aeruginosa* could be prevented by immunization with the whole cells in man (Feller, 1967) and animals (Fisher, Devlin & Gnabasik, 1969). Protection could also be induced in animals by an ethanol-precipitated fraction of the slime layer (Alms & Bass, 1965), and various culture filtrates (Jones, 1968; Johnston & Syeklocha, 1972). However, as Markley (1967) and most other investigators have pointed out, in order that immunotherapy may effectively protect against infection with pseudomonas, a vaccine should prevent infection by many strains of the bacteria. Fisher *et al.* (1969) have shown that a vaccine obtained from one strain of *P. aeruginosa*

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could protect mice against up to 21 % of other strains tested. On this basis he developed the immunotype scheme based on seven strains which protected mice against more than 90 % of the strains isolated from a wide variety of sources. In a much more closed environment, Pierson & Feller (1970) have shown that 76 % of 555 clinically isolated strains reacted with a monovalent serum.

Although numerous investigators have dealt with the antigen which could induce protection most of them have done so using one strain. It is the purpose of this study to identify the antigen which could induce protection, and to analyse its distribution among various strains. The data obtained from the *in vitro* studies were applied *in vivo* by testing the active protection due to immunization with this antigen, and passive protection due to vaccination with the antibody to this antigen.

MATERIALS AND METHODS

Bacterial strains

P. aeruginosa strains from human infections were obtained from Prof. D. Sompolinski, Assaf Harofe, Israel Government Hospital, Zriffin. The strains were maintained on tryptic soy agar (DIFCO) slants and were transferred once a week.

Preparation of vaccine

Phenol-killed vaccine (Laborde & de Fajarodo, 1965). The *P. aeruginosa* strain used for vaccine preparation was grown overnight on nutrient agar (DIFCO) at 37° C. The bacteria were harvested with 0·5 % phenolized saline and incubated in this solution overnight at 37° C. The suspension was tested for sterility and adjusted to 200 Klett units at 660 nm. in a Klett Summerson spectrophotometer. This is equivalent to 2×10^9 organisms/ml.

Alcohol precipitated fraction from slime (*APF*) (Alms & Bass, 1967). *P. aeruginosa* was grown on slants of brain heart infusion (BHI) agar (DIFCO) overnight at 37° C. The bacteria were harvested from the slant and inoculated into BHI broth. After incubation at 37° C. for 5 hr., 5 ml. of this culture were transferred to BHI agar in Roux culture bottles and incubated overnight at 37° C. The bacteria were harvested by gentle agitation with 5 ml. of 0·15 M-NaCl solution per bottle, and were then removed by centrifugation at 7300 *g* for 1 hr. The supernatant was again centrifuged at 27,000 *g* for 1 hr. to remove any debris of cells. Sodium acetate (10 % w/v) and glacial acetic acid (1 % w/v) were then added to this supernatant which contained the crude slime. The slime was precipitated by adding an equal volume of alcohol by drops at 4° C. to this solution. The alcohol-precipitated fraction of the slime was collected by centrifugation at 27,000 *g* for 1 hr. The precipitate was washed once with distilled water and then dissolved in 0·15 M-NaCl and left for 72 hr. at 4° C. This solution was afterwards dialysed for 48 hr. against distilled water and then lyophilized and stored at 4° C.

Ribosomal vaccine (Youmans & Youmans, 1966). *P. aeruginosa* was grown in a shaker water bath at 37° C. for 16 hr. The bacteria were washed twice with saline and were then suspended in 0·55 M sucrose phosphate buffer (0·01 M pH 7·0) con-

taming 0·03 M-$MgCl_2$; they were then ruptured in a cold French press. The broken cells were centrifuged at 26,300 *g* for 15 min.; four-fifths of the supernatant was transferred and spun in Spinco Model L at 46,900 *g* for 10 min. Four-fifths of the supernatant from this centrifugation was again centrifuged at 144,700 *g* for 3 hr. This supernatant was then resuspended in phosphate buffer pH 7·0, 0·01 M at a concentration of 50 mg. wet weight/ml. To a volume of ribosomal fraction an equal volume 0·5 % sodium dodecylsulphate (SDS) was added; the mixture was then left for 1 hr. at room temperature and afterwards was incubated overnight at 4° C. The SDS precipitate was removed by centrifugation at 36,000 *g* for 20 min. and four-fifths of the supernatant was again centrifuged at 144,700 *g* for 3 hr. in order to precipitate the immunizing fraction.

Mice

Female white mice of an outbreed stock were supplied by the Hebrew University colony, Jerusalem. The mice weighed between 18 and 20 *g* at the beginning of each experiment. The animals were allowed food and water freely.

Immunization procedure

Mice were injected once intraperitoneally with 0·2 ml. of either the organism suspended in phenol saline or with slime, or with ribosomal preparations. The animals were observed daily and were challenged one week later.

Preparation of challenge inoculum

Pseudomonas aeruginosa was grown on nutrient agar (DIFCO) for 24 hr. at 37° C. The bacteria were harvested from the plates, washed twice and then resuspended in saline to a concentration of 200 Klett units at 660 nm. in a Klett Summerson spectrophotometer which is equivalent to 2×10^9 organisms/ml. as determined by plate counts. Except when otherwise stated, 0·2 ml. of this suspension (10 LD50) were injected into unimmunized animals (which served as controls) and immunized animals of the same age. The unimmunized animals died within 24 hr.

Passive protection test

Sera were prepared in rabbits by immunizing them with a series of four intramuscular injections of the antigen in complete Freund adjuvant. A week after the last inoculation the animals were bled, and the sera were separated and stored at −20° C. In order to test the protective ability of the serum, the animals were injected i.p. with 0·2 ml. of serum undiluted or diluted 4 hr. before a challenge.

Antibody determination

Immunodiffusion technique. Slides 7 × 7 cm. were covered with 4 ml. of 1 % Noble agar (DIFCO). After drying at 37° C., 10 ml. of 1 % Noble agar in saline containing 0·05 % merthiolate were added. Wells were made at equal distances of 6 mm. from the central well and 0·1 ml. of antigen or antibody were added to each

well. The slides were incubated for 2 days after which they were washed for 3–4 days with saline. The precipitin line was stained with azocarmine.

Passive haemagglutination. The alcohol precipitated fraction of the slime was bound to sheep red blood cells by glutaraldehyde using a modification of the method described by Avrameas, Taudou & Chuilon (1969). Ten ml. of 2% glutaraldehyde in 0·1 M pH 7·2 phosphate saline buffer was added to 2 ml. of 50% washed sheep red blood cells in the same buffer, and the mixture was left at room temperature for 1 min. To this mixture we added 20 ml. of 0·1 M-NaH_2PO_4 which contained 0·15 M-NaCl, and this mixture was centrifuged at 5000 ***g*** for 5 min. The packed cells were then washed with the second solution and resuspended to a volume of 2 ml. (50% packed cells). Two ml. of slime (6 mg. slime/ml.) in 0·1 M-Na_2HPO_4 (pH 8·5) which contained 0·15 M-NaCl were added to these cells and this was left at room temperature for 2 hr. The sheep red blood cells which were thus coated with the slime were washed twice with phosphate buffered saline pH 7·2 and their concentration was adjusted to 2·5%.

In order to carry out haemagglutination titrations, 0·05 ml. of 2·5% sensitized cells were added to 0·5 ml. of twofold dilutions of the serum in 1% normal rabbit serum. The tubes were incubated in a water bath at 37° C. for 1 hr. and overnight in a cold room. The antibody titre was determined as the highest dilution which showed agglutination.

RESULTS

The effect of the dose and type of vaccine on the protective response of the mouse after a single intraperitoneal injection

Groups of ten mice were injected intraperitoneally with the various vaccine preparations obtained from *P. aeruginosa* strain 647. One week after vaccination the mice were challenged with 10 LD50 of the same strain and the surviving mice were counted 24 hr. afterwards. Table 1 shows that all three preparations of the antigen could protect mice against the homologous strain of *P. aeruginosa*. The various types of vaccine differed primarily in the amounts which were needed in order to produce immunization. To obtain full protection against challenge with 10 LD50 of the bacteria it was necessary to immunize a mouse with at least either 2×10^8 organisms or 100 μg. slime or 40 μg. ribosomal preparation.

Passive protection of unvaccinated mice by serum obtained from immunized rabbits

Sera obtained from rabbits immunized with the various vaccine preparations previously described, were administered intraperitoneally to the mice. Four hours after receiving the various serum dilutions the mice were challenged with 10 LD50, and the surviving mice were counted after 24 hr. Table 2 shows that the serum obtained from rabbits immunized with either the phenol-killed vaccine or the slime could afford complete protection at a 1/10 dilution, whereas the serum obtained from the rabbit immunized with the ribosomal fraction was less potent. When these various sera were examined for the presence of precipitating antibody, produced in response to the slime layer, it could be seen that this presence was

Table 1. *Protection of mice against* Pseudomonas aeruginosa *by different vaccine preparations*

Immunizing agent	Vaccine dose/ mouse	Death/ total	Survivors %
Phenol-killed bacteria	4×10^7 bacteria	7/10	30
	1×10^8 bacteria	3/10	70
	2×10^8 bacteria	0/10	100
	4×10^8 bacteria	0/10	100
Alcohol-precipitated fraction of the slime	10 μg.	8/10	20
	50 μg.	7/10	30
	100 μg.	0/10	100
	200 μg.	0/10	100
Ribosomal fraction of the bacteria	10 μg.	10/10	0
	25 μg.	7/10	30
	40 μg.	0/10	100
	75 μg.	0/10	100
Control	—	10/10	0

Table 2. *Protection conferred on mice by their passive immunization with rabbit antisera obtained against various preparations of* Pseudomonas aeruginosa *antigens*

Immunizing agent	Antiserum dilution	Presence of precipitating antibody to the slime layer	Passive haem-agglutination titre to slime	Death/ total	Survivors %
Phenol-killed bacteria	1/10	+	1/1280	0/10	100
	1/100	—	1/80	5/10	50
	1/1000	—	1/10	9/10	10
Alcohol-precipitated fraction of the slime	1/10	+	1/1280	0/10	100
	1/100	—	1/80	4/10	60
	1/1000	—	1/10	9/10	10
Ribosomal fraction of the bacteria	1/1	—	1/160	2/10	80
	1/10	—	1/10	9/10	10
Unimmunized	1/1	—	—	9/10	10

associated with complete protection. Using the much more sensitive method of passive haemagglutination, we were able to measure quantitatively the amount of antibody even in the absence of precipitating antibody. The data presented in this experiment show a relationship between the titre of antibody as measured by the latter method, and the degree of protection of the mouse against challenge with 10 LD50 of bacteria.

The effect of the alcohol-precipitated fraction of the slime on the virulence of the bacteria

Schwartzmann & Boring (1971) showed that addition of small amounts of slime to the bacteria inhibited phagocytosis as measured by phagocytic killing of the organism. When the slime was added to the bacteria and they were then

Table 3. *Effect of alcohol-precipitated fraction of the slime on the virulence of* Pseudomonas aeruginosa *to mice*

No. of organisms	LD 50	mg. slime	Death/ total	Survivors %
—	—	0·1	0/10	100
—	—	0·2	0/10	100
—	—	1·0	0/10	100
1×10^8	1	—	6/10	40
6×10^6	1/16	—	2/10	80
6×10^6	1/16	1·0	8/10	20
6×10^6	1/16	0·2	6/10	40

Table 4. *Effect of absorption by the alcohol-precipitated fraction of the slime on the protection of the mice by the immune antisera*

Antisera prepared to	Slime absorption	pHA* titre	Death/ total	Survivors %
Phenol-killed bacteria	—	1/1024	0/10	100
	+	1/10	10/10	0
Alcohol-precipitated fraction of the slime	—	1/1024	0/10	100
	+	1/10	10/10	0

* Passive haemagglutination of the slime.

injected intraperitoneally into the mice the virulence of the bacteria was increased. Table 3 shows that a small amount of slime (0·2 mg.), which by itself is not toxic and is even able to immunize the mice against challenge with the bacteria, significantly increased the virulence of the inoculum of the bacteria. An increase in the amount of slime added to the bacteria caused a further increase in virulence.

The effect of absorption of antisera by the slime fraction on their protective power

The data presented above indicated that the slime fraction when injected together with the bacteria increased the latter's virulence. The results from Table 2 would indicate that there is a relation between anti slime antibody and the degree of protection. We therefore tested the effect of absorption of the antibody by the slime layer. For this purpose 6 mg. of slime were added to 1 ml. of the serum and incubated at 37° C. for 30 min. After incubation the mixture was centrifuged at 27,000 ***g*** for 1 hr. to remove antigen-antibody complex. After centrifugation the sera were tested for remaining antibody activity by the sensitive method of passive haemagglutination. Table 4 shows that complete removal of the antibody by the slime fraction corresponded to the loss of protection afforded by the sera against challenge with 10 LD50 of the bacteria.

Specificity of the protective response of mice vaccinated with Pseudomonas aeruginosa *strain* 647

Table 5 summarizes results of experiments in which mice vaccinated with phenol-killed *P. aeruginosa* 647 were challenged after one week with 10 LD50 of

Table 5. *Specificity of the protection in mice after a single intraperitoneal injection of* Pseudomonas aeruginosa 647 *phenolized vaccine*

Strain	Precipitation with anti-slime 647	Death/ total	Survivors %
647	+	0/10	100
C-18	+	0/10	100
1179	+	0/10	100
31	+	3/10	70
352	+	4/10	60
1214	+	3/10	70
R-1634	+	4/10	60
214	+	2/10	80
M-995	−	10/10	0
F-7	+	0/10	100
1416	+	5/10	50
M-876	−	10/10	0
3444	+	3/10	70
C-11	+	0/10	100
109	+	0/10	100
119x	+	0/10	100
629	−	10/10	0
68	+	2/10	80
62–213	+	2/10	80
2185	+	0/10	100
9060	+	1/10	90
22613	+	0/10	100
643	−	10/10	0
1316	+	0/10	100

the various strains. Of the 24 strains tested complete protection was obtained against 10 strains, partial protection against 10 and no protection at all against four strains. The crude slimes obtained from these 24 strains were tested by gel diffusion against antiserum obtained by inoculation with the alcohol precipitated fraction of the slime of strain 647. We observed that only the slimes of those strains which did not show a precipitin reaction with the slime of the immunizing strain (strain 647) were able to kill all the mice which were immunized with this organism. However, complete protection was not observed against all these strains whose slime reacted with the anti-slime of the immunizing vaccine; this is probably due to the heterogeneity of the slime layer. From the results presented in Plate 1 we can see that even though there is a cross reaction between the slimes against the homologous strain we obtained two lines of precipitation. As we observed in the large group of strains tested, the probability is that there are two components of the slime, one which cross-reacts with other strains and one which is strain specific. When a larger group of strains obtained from the same hospital was tested, we found antigenic similarities to strain 647 in 121 of 130 strains tested. Further trials to identify the remaining nine strains by preparing anti-slime against one of them were unsuccessful.

Table 6. *Passive protection of unvaccinated mice with serum from rabbits vaccinated with the alcohol-precipitated fraction of slime from* Pseudomonas aeruginosa 647

			Challenging dose					
			10 LD50			1LD50		
Strain	Active protection to 10 LD50	Anti-body titre by pHA/ mouse*	serum treated animals	control	protec-tion	serum treated	control	protec-tion
647	100	1/800	0/10	10/10	100	0/10	3/10	100
629	0	1/800	10/10	10/10	0	4/10	5/10	20
1316	100	1/800	10/10	10/10	0	0/10	4/10	100
109	100	1/800	7/10	10/10	30	0/10	4/10	100
C-18	100	1/800	8/10	10/10	20	0/10	4/10	100
119x	100	1/800	10/10	10/10	0	0/10	5/10	100

*Passive haemagglutination of slime.

Passive cross-protection among Pseudomonas aeruginosa *strains with similar slime layer.*

The data presented above suggested that some degree of protection could be achieved by active immunization of mice with slime which would cross react with slime of bacteria used for challenge. When antibody prepared against the slime of *P. aeruginosa* 647 was transferred to mice (Table 6) and they were then challenged with 10 LD50 complete protection was observed only against the homologous strain. Towards other strains little or no protection at all was observed against this challenging dose, even though protection was obtained by active immunization. However, when the challenging dose was lowered protection was achieved against these heterologous strains. Even at this lower dose almost no protection was observed against a strain whose slime did not react with the antibody.

DISCUSSION

The serological heterogeneity of *P. aeruginosa* has long been recognized and several serological schemes have been proposed (Habs, 1957; Verder & Evans, 1961). However, attempts to correlate protection against challenge with the bacteria, to the various serotype schemes present were unsuccessful. Most of these investigators tried to measure agglutinating antibody (Jones, 1968) or antibody to the lipopolysaccharide fraction of the bacteria (Gaines & Landy, 1955). Recently Alms & Bass (1965, 1967*a*, *b*) have shown that protection of mice could be induced by a fraction of the slime layer. Using their results, based on the original observation of Liu, Abe & Bates (1961), we were able to show that passive protection afforded by an antiserum against challenge with *P. aeruginosa* correlated with the presence of antibody to the alcohol precipitated fraction of the slime. These antibodies were detected by the sensitive method of passive haemagglutination as developed in our laboratory. Furthermore, removal of these antibodies from the serum by absorption by the slime layer fraction caused this antiserum

to lose its protective properties for the mouse from challenge with the bacteria, even though the antiserum was prepared to the whole bacteria and not only to the slime.

Based on these observations, that antibodies to the slime layer were probably the most important factor in immunity to *P. aeruginosa* infection in mice, we developed a system based on antigenic compatibility among the various strains. The data presented in Table 5 were similar to those observed by other investigators (Fisher *et al.* 1969; Bass & McCoy, 1971; Jones, 1972) who found that active immunization with one strain might protect mice from challenge with a wide variety of serologically unrelated strains. In our system this kind of protection was related to antigenic similarity of the slime layers. Complete protection was obtained in 40% of the strains tested, which is relatively high compared to the results obtained by Fisher *et al.* (1969), who, however, got their strains from a wide variety of sources. The partial protective power of the immunizing strain against some of the strains with which their slime cross-react may be dependent on the challenging dose. When the dose was decreased a complete protection was observed also against those strains with which only partial protection had been previously obtained. However, against those strains whose slimes did not cross-react no protection at all was observed even at the level of one LD50.

The conflicting results observed in those studies which attempt to evaluate the use of gamma globulin from convalescent patients (Feingold & Oski, 1965; Stone, Graber, Martin & Kolb, 1965; Feller & Pierson, 1968) to protect against infection with *P. aeruginosa* must be assessed on the basis of two points. First, one must know if the gamma globulin used for protection has antibody against the slime layer of the bacteria which infected the patient. Secondly, the degree of infection at the time when the antiserum was administered must not be above the degree which this serum is able to handle.

Thus the presence of an immunotype scheme based on the antigenicity of the slime layers can be used for preparing a vaccine and an antiserum. The amount of antibodies to this antigen, as measured by passive haemagglutination, could be the index of the protective power of a serum against infection with *P. aeruginosa* with an antigenically similar slime layer.

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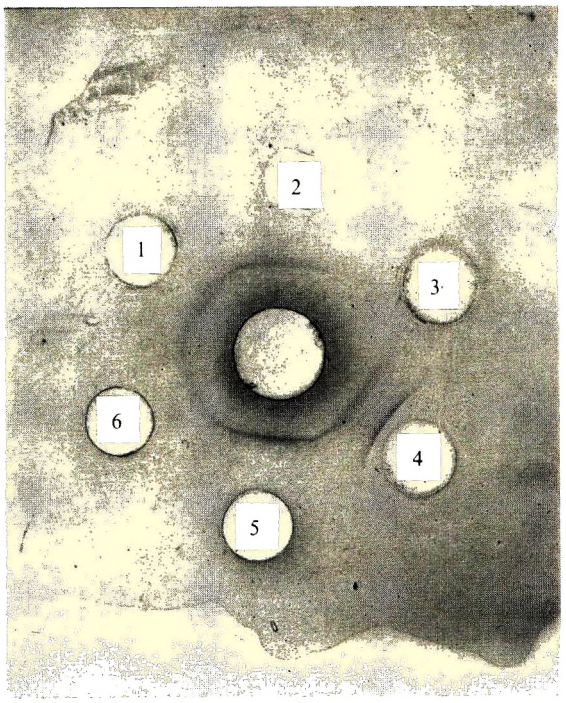
EXPLANATION OF PLATE

Immunodiffusion of rabbit anti *Pseudomonas aeruginosa* slime. In the centre, anti-*P. aeruginosa* 647 slime. In the surrounding wells slime from *P. aeruginosa* strains: 1-352; 2-214; 3-629; 4-647; 5-68; 6-109.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J. Hyg., Camb. (1974), **73**, 85
Printed in Great Britain

Attempts to infect pigs with Coxsackie virus type B5

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(*Received* 30 *November* 1973)

SUMMARY

Despite the existence of a close serological relationship between the enteroviruses Swine Vesicular Disease (SVD) and Coxsackie type B5 (Cx B5), the administration of this Coxsackie virus type to susceptible pigs by various routes failed to produce clinical disease.

Viraemia was not detected after exposure but virus was recovered intermittently from faeces and buccal swabs. A mixed virus population was demonstrated in faecal cultures from some pigs, including Coxsackie virus type B5 and other agents, presumably native pig enteroviruses. The Coxsackie virus persisted in faeces in declining amounts for up to 8 days after primary exposure.

Serum neutralizing antibody showed a transient rise to Coxsackie virus, reaching a peak at 14 days and declining below demonstrable titres by 28 days after exposure. The antibody titres attained were proportional to the dose of virus administered and the degree of neutralization was very similar to both SVD and Cx B5 viruses.

On cross challenge by exposure to SVD virus 28 days after exposure to Cx B5 virus, most animals (5/6) succumbed with typical vesicular lesions, although the serum neutralizing antibody titres showed a characteristically anamnestic response to both viruses.

INTRODUCTION

Swine vesicular disease is recognized as an emerging problem in domestic pigs. The condition has been recorded in Italy (Nardelli *et al.* 1968), Hong Kong (Mowat, Darbyshire & Huntley, 1972), the United Kingdom (Dawe, Forman & Smale, 1973) and a number of European countries, including France (Dhennin & Dhennin, 1973), Austria and Poland (Report of F.A.O. Special Meeting, 1973).

Clinically the disease is characterized by vesiculation of the feet, snout, buccal cavity and skin in a manner indistinguishable from foot-and-mouth disease. Several aspects of the pathogenesis of the disease have been studied (Burrows, Greig & Goodridge, 1973; Burrows, Mann & Goodridge, 1974; Sellers & Herniman, 1974).

Recently American workers investigated the relation between SVD virus and forty-two human enterovirus immunotypes using Lim Benyesh-Melnick serum pools (Graves, 1973). SVD virus was tested with serum raised against poliovirus types 1-3, echovirus types 1–7, 9, 11–27, 29–33, Coxsackie virus types A 7, 9, 16 and types B 1–5. The tests revealed a highly significant neutralization of SVD

virus by antiserum to Coxsackie virus type B5 and a similar effect was observed in reciprocal tests using Cx B5 virus and antiserum to SVD virus.

The striking serological relation between the porcine pathogen, SVD virus, and the human pathogen, Cx B5 virus, raised the question of other relations including the possibility of a zoonosis. This communication describes attempts to determine the susceptibility of the domestic pig to Coxsackie virus type B5.

MATERIALS AND METHODS

Animals

Pigs. Large White pigs were used weighing 30–40 kg. at the outset of the experiment. The pigs were housed in an isolation unit on concrete with straw bedding and were fed on pig meal with water *ad libitum.*

Mice. One-day-old mice of the Pirbright P (Parkes) strain of albino mice were used for the isolation and assay of virus for certain samples.

Guinea-pigs. Dunkin–Hartley strain of guinea-pigs weighing at least 500 g. were used for production of antiserum to Cx B5 virus.

Viruses

Coxsackie virus type B5. This was supplied by courtesy of Dr D. R. Gamble of the Public Health Laboratory Service, Epsom, Surrey. The virus was a prototype strain (Faulkner) originally recovered from human faeces in the United States of America and had been maintained in monkey kidney tissue culture for some 20 years. The exact passage history of the strain was unknown. The virus was administered to pigs as received and also after two further passages in pig kidney cell cultures. A glycerinated filtrate of the third pig kidney passage was stored at $-20°$ C. and used as an antigen in serum neutralization tests.

Swine Vesicular Disease virus. Strain England UK-G 27/72, recovered from a British field outbreak in 1972, was given two passages in pig kidney cell cultures before being stored as a filtrate with 50% glycerol at $-20°$ C. This stock was used as an antigen in serum neutralization tests and in the infection of donor pigs for cross-challenge purposes.

Tissue culture

The IB-RS-2 pig kidney cell line (De Castro, 1964) was used exclusively. Virus stocks were grown in Roux bottles, recovery and assay of virus from pig samples was performed in tubes incubated on a roller apparatus and neutralization tests and some infectivity titrations were carried out in petri dish cultures.

Infection of animals

Exposure of pigs to Cx B5 virus. Eight pigs (numbers KH13-20) were divided into two groups of four animals housed in the same loose box. One group was given the Cx B5 virus as originally obtained and the other received Cx B5 virus after two further passages in pig kidney tissue culture. The two inocula contained $10^{6.5}$ and $10^{7.0}$ plaque forming units (p.f.u.) per ml. respectively and Table 1 shows the route and dose of virus administered to each animal.

Table 1. *Protocol of inoculation of pigs with Cx B5 virus*

Virus	Animal number	Route of administration	Volume (ml.)	Total virus dose
Cx B5 prototype strain	KH13	Not inoculated	—	—
	KH14	Intradermal Bulbs of heel and coronary bands of all four feet	Approx 2·0	Approx 6·7*
	KH15	As for KH14		
	KH16	Subcutaneous	1·0	7·2
		Intramuscular	1·0	—
		Intravenous	1·0	—
		Intranasal	1·0	—
		Oral	2·0	—
Cx B5 prototype at 2nd IB-RS-2 passage	KH17	Not inoculated	—	—
	KH18	Intradermal Bulbs of heel and coronary bands of four feet	Approx 2·0	Approx 7·2
	KH19	As for KH18		
	KH20	Subcutaneous	1·0	8·5
		Intramuscular	1·0	—
		Intranasal	10·0	—
		Oral	20·0	—

* Log_{10} plaque-forming units.

Cross challenge of pigs with SVD virus. Twenty-five days after the first exposure of pigs to Cx B5 virus two fresh pigs (KH 56 and 57) housed in a different isolation unit were infected with SVD virus by intradermal inoculation of the coronary band and bulbs of heel using virus with a titre of $10^{6\cdot1}$ p.f.u. per ml. These donor pigs were used to contaminate two loose boxes which were not cleaned out after their introduction. Twenty-eight days after exposure to Cx B5 virus the eight pigs were transferred to the second isolation unit and there segregated into two groups of four, KH 13–16 and KH 17–20. Four fresh, non-inoculated pigs (KH 58–61) were added, two to each group, as controls for contact infection. The SVD donor animals were removed and the two groups of six pigs were then placed in the contaminated boxes.

Inoculation of mice. Mice were inoculated with buccal, pharyngeal and faecal samples from pigs by the intracerebral route with a dose of 0·03 ml. and were examined daily for the following 12 days.

Inoculation of guinea-pigs. Guinea-pigs were given 1·0 ml. of an equal mixture of Cx B5 virus at the third pig kidney cell passage and Freund's complete adjuvant. The dose was divided and inoculated intramuscularly in both hind legs. Twenty-eight days later a further 0·5 ml. of Cx B5 virus was administered intraperitoneally and after 10 days the animals were exsanguinated to provide antiserum.

Clinical examination, sampling and processing of samples

Pigs were examined clinically, their rectal temperatures recorded and samples collected from the blood, faeces, buccal cavity and occasionally the pharynx

before exposure to infection, daily for 10 days after infection and then every second or third day up to 28 days after infection with Cx B5 virus. Serum samples were collected at intervals for 22 days after cross challenge with SVD virus. Buccal swabs were taken by vigorously rubbing cotton wool buds over the oral mucosa. The swabs were shaken mechanically for 10 min. in 2·0 ml. of phosphate buffered saline (PBS) with antibiotics to give an approximately 1/10 dilution of the original sample for assay. The pharyngeal region was sampled by means of the probang cup described by Burrows (1968), the instrument being rinsed in 2·0 ml. of PBS resulting in a dilution of about 1/10. Faeces samples were taken from the rectum. One gram of faeces was shaken in 9·0 ml. of PBS as described above, the suspension was clarified by centrifugation at 3000 rev./min. for 10 min. and the supernatant was examined for the presence of virus. Blood was obtained by venupuncture from the anterior vena cava and was processed for serum in the normal manner. The majority of samples were processed and inoculated onto tissue cultures within three hours of collection. Samples were subsequently stored at 4° C. for up to 7 days or at −20° C. for longer periods.

Detection of virus and assay of infectivity

Samples were inoculated onto IB-RS-2 monolayers in tubes or Petri dishes using two cultures per dilution. Tubes were examined microscopically each day for five days. Typical enterovirus cytopathic effect (CPE) developed at about 48 hr. and increased up to 96 hr. after infection and tubes showing CPE were harvested to provide antigen for subsequent tests of viral identity. Plaque counts were made after 4 days of incubation under agar, the monolayers having been stained with methylene blue or neutral red solution.

Serological methods

Serum neutralizing antibody. Serum neutralizing antibody titre was determined by means of a plaque reduction test in IB-RS-2 monolayers using a constant virus/variable serum method. A 1/10 dilution of serum was heated at 56° C. for 30 min. before dilution in ten-fold steps from starting dilutions of 1/10 and 1/30, and 1·0 ml of each serum dilution was added to an equal volume of PBS containing approximately 300 p.f.u. of Cx B5 or SVD virus. The mixtures were incubated for 90 min. at 37° C. before inoculation in 0·2 ml. volumes onto cell monolayers. After 60 min. adsorption at 37° C., the cell sheets were overlaid with agar and returned to incubate at 37° C. for 4 days at which time the monolayers were stained and the plaques enumerated. The neutralizing antibody titre was expressed as the log reciprocal of that serum dilution which neutralized 90 % of the test virus.

Identification of porcine viral strains. All viral strains isolated from faeces and buccal swabs were examined in neutralization tests with guinea-pig antiserum to Cx B5 virus. The strains were tested as first or second tissue culture passage harvests when titration had shown that the passaged material contained $10^{6\cdot0}$ or greater p.f.u. per ml. A constant serum/varying virus dilution method was employed with 1·0 ml. of a 1/20 dilution of pooled guinea-pig antiserum heated

Table 2. *Virus content of samples derived from pigs after challenge with Cx B5 virus*

Animal number	Sample	Days after inoculation/exposure																
		0	1	2	3	4	5	6	7	8	9	10	12	14	16	18	21	25	28
KH13	BS*	0	2·0	0	2·0	0	1·5	0	0	0	0	0	0	0	1·5	0	1·0	0	0
	PH†	—	—	—	—	—	—	—	0	—	—	—	—	0	—	—	0	—	—
	F‡	0	0	1·0	2·5	4·0	0	4·5	4·5	0	2·0	2·5	0	2·5	2·0	0	0	2·0	1·5
KH14	BS	0	2·0	0	1·5	0	0	0	1·5	0	0	0	0	0	0	0	0	0	0
	PH	—	—	—	—	—	—	—	2·0	—	—	—	—	0	—	—	0	—	—
	F	0	4·0	2·0	3·0	3·0	2·5	3·0	2·5	0	0	0	0	0	0	0	0	0	0
KH15	BS	0	1·5	1·0	0	1·5	1·0	1·5	2·0	2·0	0	0	1·5	0	0	1·0	0	0	0
	PH	—	—	—	—	—	—	—	2·0	—	—	—	—	2·0	—	—	0	—	—
	F	0	3·5	0	2·0	0	2·5	2·0	2·5	2·0	1·5	0	1·5	0	0	0	0	2·5	0
KH16	BS	0	1·5	0	2·0	1·5	0	1·5	1·0	3·0	0	0	2·0	0	0	0	0	1·5	0
	PH	—	—	—	—	—	—	—	1·0	—	—	—	—	1·5	—	—	1·5	—	—
	F	0	3·0	0	4·5	3·5	3·5	3·0	2·0	3·0	0	0	2·5	0	1·0	0	1·0	0	0
KH17	BS	0	2·0	1·0	1·5	0	0	1·0	0	0	0	0	0	0	0	0	0	0	0
	PH	—	—	—	—	—	—	—	0	—	—	—	—	0	—	—	0	—	—
	F	0	3·0	3·5	3·0	3·0	0	2·0	0	2·0	0	0	0	0	0	0	0	0	0
KH18	BS	0	1·0	0	1·5	0	1·0	0	0	0	0	0	0	0	1·5	0	1·0	0	0
	PH	—	—	—	—	—	—	—	0	—	—	—	—	0	—	—	0	—	—
	F	0	0	2·5	2·2	0	2·0	0	1·5	2·0	0	0	0	1·0	0	0	1·5	0	0
KH19	BS	0	1·5	1·0	1·0	1·5	0	0	0	0	0	0	0	0	0	0	0	0	0
	PH	—	—	—	—	—	—	—	0	—	—	—	—	0	—	—	0	—	—
	F	0	3·5	3·0	3·0	0	2·0	2·0	0	1·5	0	0	0	0	0	0	0	0	0
KH20	BS	0	4·0	2·5	3·5	2·0	0	3·0	3·0	0	2·5	0	1·0	0	1·5	0	0	0	1·5
	PH	—	—	—	—	—	—	—	4·0	—	—	—	—	0	—	—	2·0	—	—
	F	3·5	5·5	5·0	3·0	1·5	4·5	0	3·0	2·5	0	1·5	0	0	2·5	0	2·5	0	0

* BS, buccal swab: infectivity expressed as TCID 50 per sample.
† PH, pharyngeal sample: infectivity expressed as TCID 50 per sample.
‡ F, faeces: infectivity expressed as TCID 50 per sample.
—, not sampled.
0, no virus detected at the lowest dilution examined.

at 56° C. for 30 min. being added to an equal volume of serial ten-fold dilutions of virus. A parallel titration was carried out with normal heated guinea-pig serum for each virus strain and infectivity was measured by the plaque technique as detailed above, a neutralization index being derived from the difference in infectivity titre of viral strains incubated with normal and immune sera.

RESULTS

Attempts to infect pigs with Cx B5 virus

In daily examinations of pigs after exposure to Cx B5 virus, the rectal temperatures remained within normal ranges and no clinical abnormalities whatsoever were detected.

Viraemia was consistently absent. Table 2 gives the virus content of faecal, oral and pharyngeal samples and shows that virus was recovered intermittently from all sites at concentrations of up to $10^{5.5}$ TCID50 per g. in faeces and $10^{4.0}$ TCID50 per sample in buccal and pharyngeal samples. The frequency and titre of viral recovery declined with time after exposure to virus. Attempts to isolate virus from porcine samples by the inoculation of one day old mice proved unsuccessful.

All buccal swab samples were free of detectable virus before the introduction of Cx B5 virus but virus was recovered from the faeces of one pig at that time. The presence of pre-existing faecal virus and the rather rapid appearance of relatively high titres of virus in faecal and oral samples derived from non-inoculated contact pigs after the introduction of experimental infection led to concern about the identity of the virus which was being recovered. Neutralization tests were carried out to determine the extent to which Cx B5 virus was involved in the recoveries and Table 3 shows the degree to which various virus strains were neutralized by guinea-pig antiserum to Cx B5 strain. The results indicated that Cx B5 virus was excreted at declining titres for up to eight days after exposure and that a considerable proportion of isolations were wholly or partly composed of other virus or viruses which were not serologically related to Cx B5 virus.

The serum neutralizing antibody response to both Cx B5 and SVD viruses is shown in Table 4. All sera were free of detectable antibody at the outset of the experiment and a transient rise in serum antibody was detected reaching a peak at 14 days and declining below demonstrable titres by 28 days after exposure to Cx B5 virus. There was some suggestion of a serum antibody response in the contact control animals which was confirmed after cross challenge.

In general the antibody titres attained were proportional to the dose of virus administered and the degree of neutralization was very similar with both Cx B5 and SVD viruses, thereby confirming the work of Graves (1973).

Cross challenge with SVD virus

The two donor pigs developed generalized disease within 48 hr. of inoculation and the amount of virus in the loose boxes was such that the non inoculated recipient control animals first showed lesions on the second and fourth day after

Table 3. *Neutralization of virus strains from pigs by antiserum to Cx B5 virus*

Animal number	Origin	Day after inoculation/exposure to Cx B5 virus on which the original isolation was made													
		0	1	2	3	4	5	6	7	8	9	10	12	14	16
KH13	BS	—	2·6*	—	2·0	—	1·7	—	—	—	—	—	—	—	0·4
	F	—	—	2·7	2·5	1·5	—	0·9	0·4	—	0·0	0·2	—	0·0	0·0
KH14	BS	—	5·0	—	⩾ 6·0	—	—	—	⩾ 6·0	—	—	—	—	—	—
	F	—	5·7	⩾ 6·0	5·9	5·7	⩾ 6·0	5·7	⩾ 6·0	—	—	—	—	—	—
KH15	BS	—	3·2	2·8	—	2·5	2·1	1·3	0·2	0·4	—	—	0·3	—	—
	F	—	2·7	—	2·0	—	2·4	0·6	0·0	0·5	0·0	0·0	—	0·3	0·2
KH16	BS	—	2·2	—	1·8	2·5	—	1·8	1·5	0·8	—	—	0·0	0·0	0·0
	F	—	3·0	—	2·4	3·6	2·0	2·5	1·0	1·0	—	—	0·2	—	0·3
KH17	BS	—	⩾ 6·0	5·7	⩾ 6·0	—	—	5·7	—	—	—	—	—	—	—
	F	—	5·7	5·9	⩾ 6·0	⩾ 6·0	—	⩾ 6·0	—	5·5	—	—	—	—	—
KH18	BS	—	1·6	—	2·0	—	0·5	—	—	—	—	—	—	—	0·4
	F	—	—	2·5	1·8	—	0·7	—	0·2	0·0	—	—	—	0·5	—
KH19	BS	—	5·7	⩾ 6·0	⩾ 6·0	5·9	—	—	—	—	—	—	—	—	—
	F	—	5·7	5·9	5·7	—	⩾ 6·0	5·6	—	⩾ 6·0	—	—	—	—	—
KH20	BS	—	3·0	3·6	3·2	2·8	—	1·7	2·5	—	0·2	—	0·4	—	0·2
	F	0·5	3·5	4·0	2·8	1·3	2·0	—	3·0	3·2	—	0·0	—	—	0·5

* Log reduction in titre of virus strain containing ⩾ 10^6 p.f.u./ml. effected by antiserum to Cx B5 virus.
BS, buccal swab. F, faeces. —, no virus isolated.

Table 4. *Results of neutralization test on the sera of pigs after challenge with Cx B5 virus*

Animal number	Days after inoculation/exposure				
	0	7	14	21	28
KH13	—/—	—/—	1·0/1·0	—/—	—/—
KH14	—/—	1·0/1·5	1·5/2·0	—/1·0	—/—
KH15	—/—	1·5/1·0	2·0/2·0	1·0/1·0	—/—
KH16	—/—	1·0/1·0	2·5/2·0	1·5/1·0	—/—
KH17	—/—	—/1·0	1·0/1·0	1·0/—	—/—
KH18	—/—	1·0/1·5	2·0/1·5	1·0/1·0	—/—
KH19	—/—	1·5/1·5	2·5/2·5	2·0/1·5	—/—
KH20	—/—	1·5/2·0	3·0/3·5	2·5/2·0	—/—

Numerator/denominator = serum neutralization titre against SVD virus/serum neutralization titre against Cx B5 virus. Results expressed as the log reciprocal of the serum dilution with neutralized 90 % of the test virus.
— = < 1·0.

Table 5. *Development of clinical disease in pigs cross-challenged with SVD virus*

Box number	Animal number	Status	Days after exposure												
			0	1	2	3	4	5	6	7	8	9	12	14	22
12	KH13	Prior exposure to Cx B5 virus	†	—	—	—	—	—	—	—	—	4*	4	4	4
	KH14		†	—	—	—	—	—	—	—	—	2	4	4	4
	KH15		†	—	—	—	—	—	—	—	—	—	—	—	—
	KH16		†	—	—	—	—	—	—	—	—	—	3	4	4
	KH58	No prior exposure	†	—	—	—	—	—	—	—	—	—	—	—	—
	KH59		†	—	—	?	1	1	1	2	2	4	4	4	4
13	KH17	Prior exposure to Cx B5 virus	†	—	—	—	—	1	1	1	1	1	1	1	1
	KH18		†	—	—	—	1	4	4	4	4	4	4	4	4
	KH19		†	—	—	1	2	2	4	4	4	4	4	4	4
	KH20		†	—	—	—	—	—	1	1	1	1	1	1	1
	KH60	No prior exposure	†	—	—	2	4	4	4	4	4	4	4	4	4
	KH61		†	—	2	4	4	4	4	4	4	4	4	4	4

*, number of feet showing lesions.
†, pigs exposed to boxes contaminated with SVD virus.
—, no lesions detected.
?, presence of an early lesion queried.
Note: on day 5, KH61 was transferred to Box 12.

being introduced to the infected boxes. The challenge was therefore adequate and the initial exposure of pigs to a contaminated environment was quickly reinforced by exposure to additional virus excreted from the recipient control animals.

Some inequality of challenge may have occurred however, since only three of the four recipient control pigs developed overt disease and lesions developed rather slowly in the susceptible pig which was paired with the clinically resistant animal. One recipient control pig from the pair showing fully generalized disease was therefore transferred into the other loose box 5 days after the initiation of challenge to ensure a more evenly matched exposure for both groups of pigs.

Table 6. *Results of neutralization tests on the sera of pigs after cross-challenge with SVD virus*

Status	Animal number	Days after exposure 0	3	7	14	22
Prior exposure to Cx B5 virus	KH13	—/—	1·0/1·5	1·5/1·5	3·5/2·0	4·0/3·0
	KH14	—/—	1·5/—	1·5/1·0	4·0/3·0	4·0/3·5
	KH15	—/—	1·5/1·0	2·0/1·0	3·0/2·5	3·5/2·0
	KH16	—/—	1·0/1·0	2·0/1·0	3·5/2·5	4·0/3·0
	KH17	—/—	1·0/1·0	1·5/1·0	3·0/2·5	3·0/2·5
	KH18	—/—	1·5/1·0	1·5/1·5	4·0/3·0	3·5/3·0
	KH19	—/—	1·0/—	1·5/1·0	3·5/3·0	4·5/3·0
	KH20	—/—	1·5/1·0	2·0/1·0	4·5/3·0	3·5/3·5
No prior exposure	KH58	—/—	—/—	—/—	2·5/2·0	2·5/2·0
	KH59	—/—	—/—	1·0/—	2·0/2·0	2·5/3·0
	KH60	—/—	—/—	1·0/1·5	2·0/2·0	2·5/3·0
	KH61	—/—	—/—	—/1·0	2·5/2·0	3·0/2·5

Numerator/denominator = serum neutralization titre against SVD virus/serum neutralization titre against Cx B5 virus. Results expressed as the log reciprocal of the serum dilution which neutralized 90 % of the test virus.

— = < 1·0.

The clinical results of the cross challenge are summarized in Table 5. Of the six pigs which were originally inoculated with Cx B5 virus, four developed typical severe vesicular lesions of all four feet, one showed mild lesions confined to a single foot and one remained free from clinical disease. Of the two non inoculated animals which had been in contact with the 6 pigs originally given Cx B5 virus, one developed fully generalized disease while the other showed lesions on one foot. As has been mentioned, one of the 4 recipient control animals in the SVD virus challenge remained free from clinical disease. The occurrence and extent of lesions was therefore variable in both control and experimental pigs but the failure of prior exposure to Cx B5 virus to confer protection against infection with SVD virus was clearly demonstrated.

The results of serum neutralizing antibody assays against both Cx B5 and SVD viruses after cross challenge are given in Table 6. Prior exposure to Cx B5 virus had sensitized both inoculated and in-contact pigs so that subsequent exposure to SVD virus resulted in a typically anamnestic response, the neutralizing antibody increasing more rapidly and to higher titres in these animals than in the control pigs which were exposed to SVD virus alone. High titre antibody was found to both viruses after cross challenge but the serum antibody response to SVD virus was higher than that to Cx B5 virus.

DISCUSSION

The failure of Cx B5 virus to produce clinical disease in pigs must be qualified by reference to a number of host and virus factors which may have influenced the outcome of the experiment.

Concurrent infection with other viruses, presumably the commonly encountered porcine enteroviruses, was demonstrated in some of the experimental animals so that interference between viruses cannot be excluded. Similarly the insusceptibility of the pigs may have been related to local or secretory defence mechanisms which were not investigated.

The prototype virus used had been maintained in tissue cultures for many years and could have altered in pathogenicity for animals during serial passage. Despite its history, however, the virus readily grew to high titre in a variety of pig organ cultures, in primary pig kidney cells and in the IB-RS-2 cell line, proving that both differentiated and de-differentiated cells of porcine origin would support the growth of Cx B5 virus *in vitro*. Moreover, cultivation of the virus in pig monolayer cells through 15 serial passages did not result in any noticeable increase in virus yield which indicated that the virus strain was already well adapted to growth in this system (A. J. M. Garland, unpublished data). The failure to recover virus from pig samples by the inoculation of one day old mice (which had been used in the hope of differentiating between Cx B5 and porcine enteroviruses) may also be explained by the high degree of tissue culture adaptation of the virus strain and indeed, in comparative titrations of Cx B5 virus infectivity, IB-RS-2 cells proved to be more sensitive than mice to the extent of $10^{3\cdot8}$ ID50.

That pigs were in intimate contact with the agent was shown by the recovery of Cx B5 virus from the alimentary tract and by the primary and secondary serum antibody responses but the virus behaved as a non-replicating antigen, being eliminated from the gut in 8 days or less and eliciting only a transient rise in serum antibody. In this context Burrows *et al.* (1973, 1974) have drawn attention to the low titres of serological response in pigs experiencing subclinical infection with SVD virus.

In a preliminary experiment Graves (1973) also found that pigs were not susceptible to Cx B5 virus. The serological results differ, however, since he found that serum antibody continued to rise for at least 21 days after exposure and attained high titre. This difference may be explained by the method of repeated instillation of virus which he employed.

In recent years there has been an increasing interest in the inter-species transfer of viral agents. Many examples are known of serological relations between viruses from different hosts and in some instances the inter-relations extend to pathogenicity. There is good evidence for the production of natural infections in man and pigs by a single antigenic subtype of influenza virus A and it has been suggested that dogs, horses and birds may also play a part in the epidemiology of the disease as disseminators of virus or as hosts in which new virus might be produced by the hybridization of human and animal strains (various authors in the *Bulletin of the World Health Organization*, 1972). Similarly, Coxsackie virus type A5 has been associated with a concurrent outbreak of virus pneumonia in pigs and acute febrile illness in children (Verlinde & Versteeg, 1958). Parainfluenza type 3 viruses isolated from a variety of animals including man, sheep and cattle are also related serologically although their different origins may be determined

by a number of serological techniques (Fischman & Bang, 1966). Each strain has been associated with respiratory disease in its respective host, but in some cases pathogenic effects have been demonstrated in other species. Thus a human strain of Parainfluenza type 3 produced pulmonary lesions in hysterectomy-produced colostrum-deprived piglets (Betts & Jennings, 1966) and bovine strains caused disease in lambs although ovine strains failed to produce disease in calves (Stevenson & Hore, 1970). In the measles–distemper–rinderpest triad, the viruses exhibit a close serological relation but in terms of natural pathogenicity they appear to be strictly species specific for their respective hosts (Imagawa, 1968).

The close serological relation between Cx B5 and SVD virus is now well established. Clinical and serological evidence indicated that SVD virus is a human pathogen since a number of workers in this laboratory became ill after exposure to the virus with symptoms which ranged from vague malaise to classical aseptic meningitis and their convalescent sera neutralized both SVD and Cx B5 viruses to high titre. Immunodiffusion studies indicated that the infection had been associated with SVD rather than Cx B5 virus (Brown, Talbot & Burrows, 1973). The zoonosis aspect must not be overemphasized since there have been no reports from the field of association between human and porcine disease during the recent widespread British SVD epidemic and there was a very low incidence of both clinical disease and serological conversion among laboratory staff directly exposed to the virus.

The present study showed that one strain of Cx B5 virus was not pathogenic for pigs, since it failed to replicate, cause disease or produce cross immunity to SVD virus when administered by a number of routes. It is possible, however, that other Cx B5 strains, recently isolated or from pig organ culture, might cause clinical disease in pigs. The use of gnotobiotic animals might facilitate such investigations by eliminating possible interference effects from other porcine enteroviruses.

The authors wish to thank Dr J. Graves who allowed us access to his results before their publication, Dr D. R. Gamble who provided the Cx B5 virus and Mr C. Hamblin and Mr G. Hutchings who rendered valuable technical assistance.

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Printed in Great Britain

Maximum likelihood solutions for the combination of relative potencies

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(*Received* 3 *December* 1973)

SUMMARY

Two published methods for the maximum likelihood estimation of a common relative potency from a series of parallel-line assays are shown to be equivalent.

Bennett (1962) and Armitage (1970) independently published methods of obtaining a maximum likelihood estimator of a common relative potency from a series of parallel-line assays. The formulae in the two papers look quite different, and the reader is tempted to conclude that the assumptions underlying the two methods must differ in some obscure but important way. There are minor differences in assumptions, but these are quite unimportant. We show below that the two methods are algebraically equivalent and therefore give the same numerical results.

The first hurdle is to overcome the notational differences of the two papers. In the present paper we use a compromise notation which differs only a little from the other two systems.

Suppose that there are k parallel-line assays, and that in the ith of these a typical observation (the jth) on the standard preparation gives response y_{ij} at log-dose x_{ij}, and similarly the j'th observation on the test preparation gives response $y'_{ij'}$, at log-dose $x'_{ij'}$. Since the regressions of response on log-dose are assumed linear and parallel, the expected value $E(y_{ij})$ is

$$E(y_{ij}) = \alpha_i + \beta_i x_{ij}, \tag{1}$$

and, similarly,

$$E(y'_{ij'}) = \alpha'_i + \beta_i x'_{ij'}. \tag{2}$$

Let the log-potency of the test preparation in terms of the standard be μ. Then

$$\alpha'_i = \alpha_i + \beta_i \mu. \tag{3}$$

The problem is to estimate the parameters α_i, β_i and μ from the data by maximum likelihood. Bennett assumes that the distribution of the observations about their regression lines is normal, with constant variance σ^2. As is well known,

the maximum likelihood solution is then the same as that given by least squares, i.e. one seeks the values of the parameters which minimize the sum of squares of residuals of the y's from the fitted lines.

Bennett considers *two* residual sums of squares of y's: (i) Q_a, the minimum residual from k pairs of parallel lines with equations (1) and (2), but *without* the assumption (3) of constant potency ratio; (ii) Q_r, the minimum residual from pairs of parallel lines obeying the restriction (3), i.e. the required solution.

Now, if we assume a common potency μ, and if μ_0 is any estimator of μ, the best fit for the ith assay, consistent with the value μ_0, will be obtained by sliding the results for the test preparation along the log-dose axis by a distance μ_0, and fitting a single regression line for both preparations. That is, for the ith assay, given μ_0, we should fit a single line to the sets of observations

$$\{x_{ij}, y_{ij}\} \quad \text{and} \quad \{x'_{ij'}+\mu_0, y'_{ij'}\}. \tag{4}$$

The residual sum of squares of the y's, summed over the k assays, would give Q_r.

For Q_a, on the other hand, we should fit two parallel lines to the two sets of observations (4), with no restriction about the distance between them; suppose the slope is B_i. The difference $Q_r - Q_a$ is then the sum of k contributions, one for each assay, and it is well known from the analysis of covariance for two groups that the contribution for the ith assay, R_i, is

$$R_i = \frac{\{\bar{y}'_i - \bar{y}_i - B_i(\bar{x}'_i + \mu_0 - \bar{x}_i)\}^2}{\frac{1}{N'_i} + \frac{1}{N_i} + \frac{1}{S_{ixx}}(\bar{x}'_i + \mu_0 - \bar{x}_i)^2}. \tag{5}$$

The quantity inside the main brackets in the numerator of R_i is the vertical distance between the two lines fitted for Q_a; the numerator is the appropriate multiplier of σ^2 in the variance of this vertical distance.

For the desired solution, we must minimize $Q_r - Q_a = \sum_{i=1}^{k} R_i$ with respect to μ_0, giving the maximum likelihood estimator $\mu_0 = \hat{\mu}$. This quantity to be minimized is (apart from a multiplying factor) Bennett's equation (5); see also lines 4–6 above his (7). The minimization is not a straightforward algebraic problem, and Bennett does not discuss the procedure in detail.

It is convenient now to introduce some simplified notation. Write*

$$D_i = \bar{y}'_i - \bar{y}_i,$$

$$z_i = \bar{x}_i - \bar{x}'_i,$$

$$v_i = \frac{1}{N_i} + \frac{1}{N'_i},$$

and

$$u_i = \frac{1}{S_{ixx}}.$$

* This is almost the notation of Armitage (1970). His u_i and v_i are equal to the quantities defined above multiplied by σ^2, but, as he points out, a knowledge of σ^2 is not required for the solution, and this parameter is better omitted from the argument.

Then the quantity to be minimized, ΣR_i, is, from (5),

$$\Sigma \frac{\{D_i - B_i(\mu_0 - z_i)\}^2}{v_i + u_i(\mu_0 - z_i)^2}. \tag{6}$$

Differentiating (6) with respect to μ_0, equating to zero at $\mu_0 = \hat{\mu}$, and simplifying, gives

$$\Sigma \frac{\{v_i B_i + u_i(\hat{\mu} - z_i)\, D_i\}\, \{D_i - B_i(\hat{\mu} - z_i)\}}{\{v_i + u_i(\hat{\mu} - z_i)^2\}^2} = 0. \tag{7}$$

Now, compare (7) with the two equations required by Armitage's (1970) iterative solution (his equations (1) and (2), called here (A1) and (A2)):

$$\hat{\beta}_i = \frac{\lambda_i B_i + (\hat{\mu} - z_i)\, D_i}{\lambda_i + (\hat{\mu} - z_i)^2}, \tag{A1}$$

$$\hat{\mu} = \Sigma \frac{(D_i + z_i \hat{\beta}_i)\, \hat{\beta}_i}{v_i} \Big/ \Sigma \frac{\hat{\beta}_i^2}{v_i}. \tag{A2}$$

These involve the maximum likelihood estimator $\hat{\beta}_i$ of β_i, which has not previously entered the argument. However, (A1) and (A2) are together equivalent to (7). For if $\hat{\beta}_i$ from (A1) is substituted in (A2), and the result simplified, (7) is obtained. The two solutions are equivalent. To put this another way, (A1) and (A2) provide a relatively simple iterative system for solving (7); their derivation is particularly simple (Armitage, 1970) because one may proceed from the distributions of B_i and D_i without going back to the original observations (x_{ij}, y_{ij}) and $(x'_{ij'}, y'_{ij'})$.

One advantage of Bennett's approach is that it leads to the calculation of confidence regions for μ. If μ_0 were the true value of μ, (6) would be distributed as σ^2 times a χ^2 variate on k degrees of freedom. If σ^2 is known, equating (6) to σ^2 times certain percentiles of this χ^2 distribution will give the appropriate confidence region for μ. The same solution is obtained by deriving a generalized likelihood ratio χ^2 statistic from the expression for log likelihood given by Armitage. If σ^2 is not known, a similar procedure is followed with the F distribution.

Another approach to the derivation of confidence regions for μ is to use a χ^2 variate on 1 degree of freedom, representing the improvement in fit due to using the maximum likelihood estimator $\hat{\mu}$ rather than an arbitrary value μ_0. These different approaches will be discussed further in a forthcoming joint paper with D. J. Finney.

We are grateful to Professor D. J. Finney for suggesting that the relationship between the two solutions should be explored.

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J. Hyg., Camb. (1974), **73**, 101
Printed in Great Britain

Swine vesicular disease: attempts to transmit infection to cattle and sheep

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(*Received* 2 *January* 1974)

SUMMARY

Cattle and sheep were housed with infected pigs for 11 days. Small amounts of virus were recovered intermittently from the pharynx, milk and rectal swabs of the cattle, but no evidence of subclinical infection was found. Some indication of virus growth in the sheep was obtained in that large amounts of virus were recovered from the pharyngeal region 4 to 7 days after exposure and six of the eight sheep developed significant titres of neutralizing antibody which were maintained in four animals for at least 6 weeks.

INTRODUCTION

The host range of swine vesicular disease virus (SVDV) appears to be limited to pigs and infant mice (Nardelli *et al.* 1968) and man (Brown, Talbot & Burrows, 1973). Nardelli *et al.* (1968) failed to produce signs of infection in cattle, donkeys, rabbits, guinea-pigs and hamsters with the Italy/66 strain and Dawe, Forman & Smale (1973) and Dhennin & Dhennin (1973) confirmed that the UK/72 and France/73 strains of virus did not produce lesions following intradermal tongue inoculation of cattle and calves. During the 1972/73 outbreak of swine vesicular disease (SVD), cattle and sheep were in close contact with large numbers of infected pigs on several farms. Although observations in the field (R. S. Hedger, personal communication) indicated that cattle and sheep were unlikely to be of importance in the epizootiology of the disease, experiments were carried out to study the response of these species to a prolonged and intimate exposure to infected pigs.

MATERIALS AND METHODS

Virus

The England/72 virus (Dawe *et al.* 1973) was used as a suspension of infected pig foot epithelium for animal inoculation and as a tissue culture harvest from the second passage in the pig kidney cell line IB-RS-2 (de Castro, 1964) for neutralization tests.

Experimental animals

Pigs – inoculation and sampling procedures. Eight Large White pigs (30 to 40 kg) were inoculated in both heels of both fore feet with $10^{5.9}$ p.f.u. of virus at each site. Forty-eight hours after inoculation the pigs were moved into animal rooms containing cattle or sheep and left there for 11 days. Two pigs were placed with each of three cattle and two pigs with eight sheep. Nasal, oral, rectal, preputial or vaginal swabs, blood and pharyngeal/tonsillar samples were taken daily for 12 days from the donor pigs (Burrows, Mann & Goodridge, 1974).

Cattle. Two aged Friesian cows, one in late lactation and one in middle lactation, and one 2-year-old cross-bred Devon steer were housed in separate boxes. The cows were milked by hand once or twice daily and samples of pooled fore milk and bulk milk were taken from each cow at each milking session. Oesophageal/pharyngeal samples (Burrows, 1966) and rectal swabs were taken daily. Five days after the removal of the pigs the milking cows were re-exposed to infection by the instillation of $10^{7.0}$ p.f.u. of virus into one quarter of the mammary gland (Burrows *et al.* 1971).

Sheep. Eight cross-bred sheep were housed in one room. Rectal swabs and pharyngeal samples (Burrows, 1968) were taken daily from four of the sheep.

Assay of virus and neutralizing antibody

Samples were stored, prepared and assayed for virus and neutralizing antibody as described by Burrows *et al.* 1974.

RESULTS

Pigs

All pigs developed primary lesions within 48 hours and secondary lesions within 3 to 4 days. Details of the amounts of virus found in the daily samples collected from these animals have been recorded (Burrows *et al.* 1974). Peak concentrations of virus were found in samples from the third to the fifth day after inoculation. The infectivity declined after the fifth day and relatively few isolations of virus were made from the swabs after the eighth day. However, virus was excreted in the faeces for longer periods. The mean virus content of faeces collected 6 days after inoculation was $10^{4.8}$ p.f.u./g. and $10^{2.8}$ p.f.u./g. in samples collected on the 14th day.

Cattle

No clinical evidence of disease was seen. Table 1 lists the amount of virus found in the oesophageal/pharyngeal samples, in rectal swabs and in milk during the period that the cattle were exposed to the infected pigs. These amounts were small in relation to those found in similar samples from infected pigs and the variations in the appearance and amounts of virus were not indicative of virus multiplication in the cattle. Virus inoculated into the mammary gland disappeared rapidly. Approximately $10^{4.0}$ p.f.u./ml. were found in milk collected 6 hr.

Table 1. *Recovery of virus from cattle housed with infected pigs*

Days after exposure	Steer KE 50		Cow KE 52			Cow KE 53		
	Pharynx	Rectum	Pharynx	Rectum	Milk	Pharynx	Rectum	Milk
0	0	0	0	0	0	0	0	0
1	2·0*	1·7	2·2	2·5	0	0	2·3	0
2	2·6	2·6	2·0	2·0	0·9	0	1·9	0
3	2·4	1·2	1·7	2·5	0·8	0	1·7	0
4	2·0	1·5	2·3	3·2	0	0	1·8	0
5	0	2·4	0	1·2	0	0	1·9	1·0
6	2·0	0	0	1·8	0	0	0	0
7	1·7	0	0	1·7	0	0	0	0
8	0	2·8	0	2·1	0	0	0	1·2
9	2·6	1·7	0	0	0	0	0	0·7
10	0	2·0	0	0	0	0	1·2	0
11	0	1·5	0	0	0	0	1·2	0

* Log_{10} p.f.u./sample or swab/ml. milk.
0 = < 0·3 p.f.u./ml. (milk), < 1·7 p.f.u./sample (pharynx), < 1·2 p.f.u./swab (rectum).

after instillation of $10^{7·0}$ p.f.u., $10^{1·5}$ p.f.u./ml. after 24 hr. and $10^{0·9}$ p.f.u./ml. (one cow only) after 48 hr. No virus was recovered in the milk collected 56 and 72 hr. after instillation. Slight increases in the virus-neutralizing activity of sera were detected during the course of the experiment but these increases were not as great as those found in subclinical infections of pigs (Burrows *et al.* 1974).

Sheep

No obvious signs of disease attributable to SVDV were seen. Table 2 lists the amounts of virus recovered from each sample taken from four of the eight sheep, the daily geometric mean virus content of these samples and, for comparison, the daily geometric mean amounts of virus found for all samples taken from the two donor pigs. Although the amounts of virus recovered from these pigs decreased from the first day of contact onwards, the amounts of virus recovered from the sheep increased. Maximum concentrations of virus were recovered from the pharyngeal region of three sheep on the fourth day and from one animal on the seventh day. The amounts of virus in rectal swabs varied but the highest mean amount was recorded on the sixth day of contact. Virus was not recovered from the pharynx after 8 days or from rectal swabs after 9 days.

Eleven days after their first exposure to infected pigs, the sheep were moved to a clean room and held there for a further 5 weeks. Samples of fresh faeces were examined on four occasions and virus was recovered from one animal 8 days and from three sheep 15 days after their last contact with the infected pigs (Table 3). The serum neutralizing antibody responses of the individual sheep are listed in Table 4. Six of the eight sheep developed significant antibody titres (> 1·5) and these were maintained in four animals for at least 6 weeks.

Table 2. *Recovery of virus from sheep housed with infected pigs*

Days after exposure	KE 42		KE 43		KE 44		KE 45		Geometric means		Donor pigs
	Pharynx	Rectum	Pharynx	Rectum	Pharynx	Rectum	Pharynx	Rectum	Pharynx	Rectum	
1	2·4*	1·8	3·2	1·9	2·2	2·5	2·8	2·7	2·6	2·2	5·7†
2	—	3·9	5·5	3·3	2·3	2·9	2·8	2·8	2·6	3·2	4·9
3	1·7	3·0	5·9	2·4	3·4	2·7	3·5	3·5	3·6	2·9	4·4
4	5·0	2·4	6·1	2·5	2·8	2·7	3·7	2·5	4·4	2·5	3·5
5	3·8	2·8	4·5	3·0	3·7	2·1	2·9	2·1	3·7	2·5	2·3
6	3·1	3·9	3·1	2·8	4·8	3·8	1·7	2·7	3·2	3·4	0·6
7	2·0	3·5	3·3	3·7	5·0	4·7	2·0	—	3·1	3·0	0·5
8	—	1·7	—	—	1·7	—	—	1·5	0·4	0·8	—
9	—	1·7	—	—	—	—	—	—	—	0·4	—
10	—	—	—	—	—	—	—	—	—	—	Not tested
12	—	—	—	—	—	—	—	—	—	—	—

* Log_{10} p.f.u./sample (Pharynx) or swab (rectum).

† Geometric mean amounts of virus (log_{10} p.f.u.) recovered from oral, nasal, rectal and preputial swabs and pharyngeal samples.

— = < 1·2 p.f.u./per sample or swab.

Table 3. *The recovery of swine vesicular disease virus from the faeces of* 8 *sheep after their removal from an infected environment*

Days after last exposure to infected pigs	Number of samples containing virus	Virus content
8	1/5*	2·8†
14	0/8	
15	3/8	1·0, 1·0, 1·8
22	0/8	

* Number of samples from which virus was recovered/number of samples collected.
† Log_{10} p.f.u./g.

Table 4. *Neutralizing antibody response of cattle and sheep exposed to infected pigs*

Species	Identification	Exposure	Days after exposure: 0	11	22	31	44
Steer	KD 50	Contact	—	1·5*	1·5	Experiment discontinued	
Cow	KD 52	Contact and intramammary instillation after 16 days	—	1·2	1·0	Experiment discontinued	
Cow	KD 53		—	1·5	1·0		
Sheep	KD 42	Contact with daily sampling	—	2·0	1·5	1·3	1·3
	KD 43		—	2·0	1·8	1·8	2·2
	KD 44		—	1·8	1·8	1·5	1·8
	KD 45		—	3·0	2·0	3·0	2·5
Sheep	KD 46	Contact only	—	1·3	1·8	1·8	1·5
	KD 47		—	1·0	1·1	1·3	1·3
	KD 48		—	1·8	2·0	1·8	2·5
	KD 49		—	0·8	1·3	1·5	1·0

* Log reciprocal of the serum dilution which neutralized 90 % of test virus.
— = < 0·7.

DISCUSSION

Although the three cattle acquired considerable amounts of virus from the infected environment, no evidence of active infection was obtained. The individual variations in the frequency and amounts of virus found in the samples collected from the cattle were believed to be due to differences in cleaning procedures adopted in the animal rooms and to differences in behavioural patterns exhibited by each group of animals. No bedding was provided for steer KE 50 and the floor of the room was washed and brushed each morning immediately before examination and sampling. This procedure is likely to produce aerosols of virus present on the floor or in pig faeces and this could explain the consistent pattern of virus recovery from this animal. Straw bedding was provided for the two milking cows and cleaning was restricted to the removal of faeces and soiled straw. Cow KE 53 accepted the pigs and showed little interest in them; no virus was recovered from pharyngeal samples and consequently only small amounts of virus were found in the rectal swabs. Cow KE 52 objected to the presence of the pigs and a corner of the room was fenced to enable the pigs to escape from her attentions. This interest

in the pigs may be the explanation for the greater amounts of virus found in the pharyngeal samples and the rectal swabs from this cow. Virus was recovered sporadically and in small amounts from the milk of both cattle and this was almost certainly due to contamination during the milking procedures, as no virological or serological evidence of virus growth was obtained following the instillation of virus into the mammary gland.

In contrast to the findings for cattle, evidence was obtained of virus growth in the sheep. The virus content of pharyngeal samples increased over a period of 4 to 7 days, although during this period the amounts of virus excreted by the donor pigs declined. Virus concentrations of $10^{5\cdot0}$ to $10^{6\cdot1}$ p.f.u. were found in samples from three of the four sheep. These amounts were as large as those found in pharyngeal samples taken from contact pigs 2 to 5 days after a similar exposure (Burrows *et al.* 1974).

Significant titres of neutralizing antibody ($> 1\cdot5$) were found in five of the eight sheep within 11 days and these titres were maintained in four of the animals for at least 6 weeks. It had been appreciated in the design of the experiment that repeated sampling from the pharynx might introduce passively acquired virus into the epithelium of the area and so mimic vaccination. Lower antibody titres were found in the group which had not been subjected to pharyngeal sampling but significant titres developed in two animals.

The appearance of small quantities of virus in the faeces of sheep some time after they had been removed from an infected environment was unlikely to have been due to continued virus growth in the animal. No attempt had been made to wash or disinfect the sheep and it is likely that the fleece was heavily contaminated with virus. Self-grooming activities could explain the intermittent appearance of small quantities of virus in the faeces.

These findings confirm that cattle are unlikely to be of importance in the epizootiology of SVD apart from acting as mechanical transporters of virus. The role of sheep, however, is less clear. Although the results indicate that some sheep can acquire subclinical infections when exposed to large amounts of virus for prolonged periods, this situation is unlikely to arise under normal farming conditions. The importance of sheep in the epizootiology of SVD may depend on whether or not they can acquire infection from grazing contaminated pastures, and whether or not infection can spread from sheep to sheep or from sheep to pig under natural conditions.

We should like to thank Mrs Jean Huntley and Mr G. Hutchings for valuable assistance in the laboratory and in the Isolation Unit.

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J. Hyg., Camb. (1974), **73**, 109
Printed in Great Britain

Swine vesicular disease: comparative studies of viruses isolated from different countries

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(*Received* 1 *February* 1974)

SUMMARY

Seven viruses isolated from outbreaks of swine vesicular disease in various countries between 1966 and 1973 were compared in pigs and infant mice. All produced a similar disease and virus excretion pattern in the pig, although the Italy/66 virus was considerably less virulent than the other viruses. The results of cross neutralization tests of convalescent pig sera and the response of 5-day-old mice to intraperitoneal inoculation indicated minor differences between some viruses. The Italy/66, Hong Kong/71 and France/73 viruses differed from each other and also from the Italy/72, England/72, Austria/73 and Poland/73 group of viruses.

INTRODUCTION

Outbreaks of swine vesicular disease (SVD) were recognized in a number of European countries during the autumn and winter of 1972/1973. Previous outbreaks of disease had been identified in Italy in 1966 and in Hong Kong in 1971. This sudden appearance of a relatively new disease simultaneously in several countries suggested that infection had been introduced from a common source and that the same agent was responsible. However, obvious differences in the nature and the spread of the disease were reported from different countries. These apparent differences in epizootiology may have been due to regional differences in the methods of husbandry and marketing of pigs or to differences in the virus strains isolated in the various outbreaks. This paper is concerned with some comparative studies of SVD viruses isolated from different countries.

MATERIALS AND METHODS

Viruses

The following viruses: Italy/66 (Nardelli *et al.* 1968), Hong Kong/71 (Mowat, Darbyshire & Huntley, 1972), England/72 (Dawe, Forman & Smale, 1973), Italy/72, Austria/73, Poland/73 and France/73 – were used as suspensions of infected pig foot epithelium or as tissue culture harvests from an early passage in either primary pig kidney monolayers or in the pig kidney cell line, IB-RS-2 (de Castro, 1964).

Experimental animals

Pigs: inoculation, examination and sampling procedures

Experiments with Hong Kong/71, Italy/72, Austria/73, Poland/73 and France/73 viruses. Groups of four Large White pigs (30–40 kg.) were housed in different rooms in an isolation unit and exposed to a range of virus concentrations by heel inoculation (Burrows, 1966). A group of four uninoculated pigs were included as controls. All animals were examined daily, rectal temperatures recorded and oral swabs collected for virus excretion studies. The severity of the clinical disease was assessed periodically by computing a lesion score based on the appearance and severity of lesions at predilection sites on the coronary bands, heels, accessory digits, skin, snout and mouth. A pig exhibiting extensive lesions involving all susceptible sites qualified for a lesion score of 100.

Experiments with the Italy/66 and England/72 viruses. Details of these experiments have been recorded (Burrows, Greig & Goodridge, 1973; Burrows, Mann & Goodridge, 1974).

Mice

Litters of 5-day-old mice were inoculated by the intraperitoneal route with 0·1 ml. of various concentrations of each virus and observed daily for 3 weeks. Deaths and paralysis in mice given small amounts of virus were regarded as specific only if large amounts of virus were recovered from the carcase. Survivors were killed after 6 weeks and subclinical infections were identified by serum neutralization tests.

Assay of virus and neutralizing antibody

Virus was assayed by counts of plaque-forming units (p.f.u.) after 48 hr. incubation on IB-RS-2 monolayer cultures. Serum neutralization tests were performed as described by Burrows *et al.* (1973), using a plaque reduction procedure in which residual virus was determined after 48 hr. incubation. Cross-neutralization products were calculated as described by Federer, Burrows & Brooksby (1967).

RESULTS

Pigs

Response to heel inoculation

There was little correlation between the dose of virus inoculated into the heel and the frequency of lesions produced and it was not possible to determine the amounts of virus necessary to produce a 50% lesion endpoint (Table 1). The total number of sites reacting to the inoculation of $10^{3\cdot5}$ to $10^{6\cdot4}$ p.f.u. ranged from approximately 12% with the Italy/66, Hong Kong/71 and Italy/72 viruses and 20% with the France/73 virus to between 45 and 63% with the England/72, Austria/73 and Poland/73 viruses. With these last three viruses a 100-fold increase in the amounts of virus inoculated resulted in only a mean 18% increase in the number of sites reacting.

Table 1. *Response of pigs to heel-inoculation of various concentrations of SVD virus strains*

Virus dose*	Italy/66	Hong Kong/71	Italy/72	England/72	Austria/73	Poland/73	France/73
6.5	11/16†	—	—	—	—	—	—
5.5–6.4	3/8	1/8	0/8	22/36	6/8	5/8	3/8
4.5–5.4	1/28	1/8	1/8	5/8	7/8	2/8	0/8
3.5–4.4	—	1/8	2/8	6/10	2/8	4/8	2/8
No. of pigs	17	4	4	15	4	4	4

* Log_{10} p.f.u./site.
† Number of sites reacting at 72 hr./number of sites inoculated.
— Not tested.

Table 2. *Lesion scores of pigs following heel-inoculation of SVD virus strains*

Days after inoculation	Hong Kong/71	Italy/72	Austria/73	Poland/73	France/73
8	47 (25–68)*	45 (21–70)	55 (49–60)	39 (17–52)	52 (39–63)
14	37 (21–46)	32 (15–62)	56 (46–66)	33 (16–43)	52 (39–77)
21	33 (18–47)	26 (11–45)	49 (37–58)	33 (15–35)	53 (38–73)
28	28 (11–40)	24 (8–39)	40 (27–51)	18 (3–27)	37 (22–65)

* Mean and range of four pigs.

Table 3. *Virus content of buccal swabs taken from pigs after heel inoculation of SVD virus strains*

Days after inoculation	Hong Kong/71	Italy/72	England/72	Austria/73	Poland/73	France/73
2	5·57*	4·17	4·0	3·62	5·52	3·07
3	5·17	6·00	6·30	5·95	5·92	5·22
4	5·60	6·10	6·0	7·40	5·85	6·95
5	5·25	5·72	5·14	5·65	4·67	4·81
6	3·77	3·15	4·19	3·47	3·60	3·30
7	2·20	2·55	0·99	1·32	1·80	1·57
8	2·10	0·75	—	1·92	0·37	0·87
9	1·22	1·02	—	1·37	1·35	—
10	0·67	—	—	0·67	—	0·55
11	0·85	0·30	—	1·46	—	—
12	0·42	0·37	—	—	0·37	0·37
13	—	—	NT	—	—	—
14	NT	NT	—	NT	NT	NT
19	—	—	NT	—	—	—
22	—	—	NT	—	—	—

* Log_{10} p.f.u./swab – geometric mean of four pigs (England/72 = geometric mean of eight pigs).
– = < 0·25 (< 0·12 for England/72 group). $10^{1·0}$ p.f.u./swab is the smallest amount of virus which could be detected by the method used in a single swab, but the figures in the table are means of four or eight animals.
NT = Not tested.

Table 4. *Neutralizing antibody titres of convalescent pig sera (28 day) to homologous and heterologous strains of SVD virus*

	Virus strain						
Serum	Italy/66	Hong Kong/71	Italy/72	England/72	Austria/73	Poland/73	France/73
Italy/66	3·76*	2·92	3·30	3·30	3·10	3·36	3·30
Hong Kong/71	3·04	3·13	3·30	3·30	2·97	3·10	2·94
Italy/72	3·31	3·02	3·36	3·53	3·08	3·30	3·20
England/72	3·94	3·54	3·87	3·94	3·94	3·53	3·59
Austria/73	3·52	2·70	3·64	3·36	3·19	3·70	3·30
Poland/73	2·97	2·86	3·10	3·21	2·64	3·10	3·04
France/73	3·47	2·94	3·47	3·21	3·02	3·47	3·64

* Log_{10} reciprocal of initial serum dilution which neutralized 90 % of test virus – geometric mean of three tests.

All seven viruses produced a similar disease which was characterized by a febrile episode of 3–7 days' duration with group mean peak temperatures of 39·1° C to 41·1° C. on the fourth to sixth day after inoculation. The severity of the generalized disease was not necessarily related to the initial response to inoculation. Some pigs did not develop vesicles at the sites of injection but subsequently developed secondary lesions. Table 2 lists the means and ranges of lesion scores over a period of 28 days for the pigs used in the comparative examination of five of the seven viruses. Differences were seen in the distribution and severity of vesicular lesions but the variations between individuals within a group were as great as those seen between groups. The clinical records for earlier experiments with the Italy/66 and England/72 viruses were not detailed sufficiently to compute comparable lesion scores but they do confirm that, when tested in pigs, the England/72 virus was of similar virulence to, and the Italy/66 virus was considerably less virulent than, the viruses listed in Table 2.

Virus content of buccal swabs

Table 3 lists the geometric mean amounts of virus recovered from buccal swabs taken from six of the seven groups of pigs. In general, similar amounts of virus were found in each group, the greatest amounts being found on the third or fourth day after inoculation. Virus was not recovered from the England/72 group after the seventh day but small amounts were recovered from individual pigs of the other groups on the 10th to the 12th day.

Serological studies

Table 4 presents the neutralizing antibody titres of pooled 28-day convalescent pig sera to the homologous and heterologous viruses. Each result is the mean of three separate tests and the standard error of these means, calculated on the 147 paired results, is $\pm 0{\cdot}16$. The cross neutralization products derived from these results are listed in Table 5. The differences found are not sufficiently great to

Table 5. *Inter-relationships of SVD virus strains – cross-neutralization products of convalescent pig sera*

Italy/66						
0·93	Hong Kong/71					
0·51	0·17	Italy/72				
0·46	0·23	−0·10	England/72			
0·33	0·65	−0·17	−0·17	Austria/73		
0·53	0·27	0·06	0·30	−0·05	Poland/73	
0·63	0·89	0·33	0·78	0·51	0·23	France/73

Cross-neutralization product = (A − B) + (C − D), where A and C are antibody titres (Log reciprocal) against the homologous virus strains and B and D are antibody titres against the heterologous virus strains.

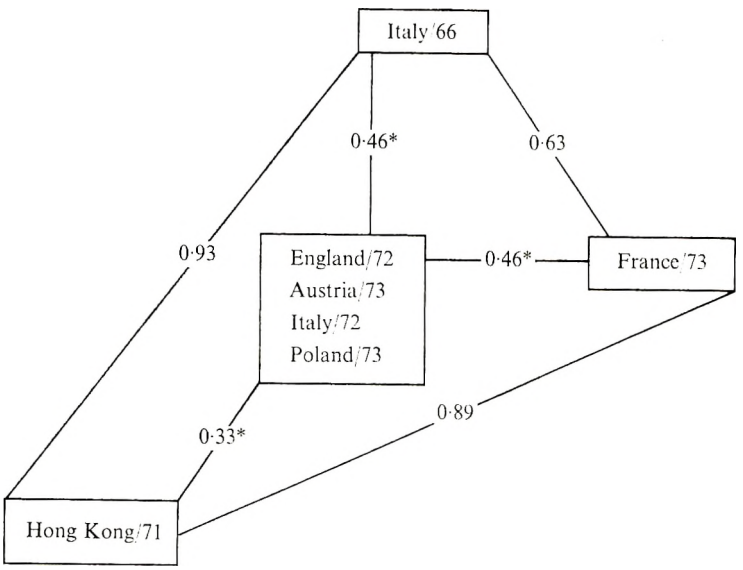

Fig. 1. Inter-relationships between SVD virus strains – cross neutralization products of convalescent pig sera. * Geometric means. Geometric mean of relationships between England/72, Austria/73, Italy/72 and Poland/73 = 0·14.

warrant a subtype classification but they do indicate minor antigenic differences between certain viruses. The England/72, Italy/72, Austria/73 and Poland/73 strains appear to be very closely related and slightly different from the other three strains, which also differ slightly from each other. A semi-diagrammatic representation of the serological inter-relationships between these viruses is shown in Fig. 1.

Table 6. *The infectivity of Italy 66 virus for one-day old mice*

Route of inoculation	Number of tests	Geometric mean infectivity	Range
Intracerebral	5	3·10*	1·7–3·8
Intraperitoneal	2	4·05	3·3–4·8

* Log_{10} p.f.u. producing paralysis or death in 50 % of mice.

Table 7. *Response of 5-day-old mice to the intraperitoneal inoculation of various concentrations of SVD virus strains*

Virus dose*	Italy/66	Hong Kong/71	Italy/72	England/72	Austria/73	Poland/73	France/73
5·5–6·5	0/10†	—	2/10	1/10	9/10	6/10	0/10
4·5–5·4	8/18	0/10	6/19	11/19	5/19	8/18	1/18
3·5–4·4	0/19	0/18	4/19	7/18	3/19	14/20	4/19
2·5–3·4	2/18	0/19	7/20	9/18	5/20	10/18	0/20
1·5–2·4	0/19	0/20	1/19	3/20	9/20	5/19	0/20
0·5–1·4	0/20	0/19	0/20	4/19	1/20	1/18	0/20
Totals:	10/104	0/86	20/107	35/104	32/108	44/103	5/107

* Log_{10} p.f.u.
† Number of mice dead or paralysed/number of mice inoculated.

Infectivity for mice

In earlier studies of the Italy/66 virus, it had been found that one-day-old mice were susceptible to both intracerebral and intraperitoneal inoculation of virus and that 50 % end-points could be calculated on the numbers of mice which developed paralysis or died (Table 6). No apparent disease was seen in 7-day-old mice given similar amounts of Italy/66, but Dawe *et al.* (1973) reported that the England/72 virus produced paralysis and death in 6-day-old mice. The results of two experiments comparing the response of 5-day-old mice to the intraperitoneal inoculation of the seven viruses are listed in Table 7. The numbers of mice showing signs of disease differed considerably; less than 10 % of mice given the Italy/66, Hong Kong/71 and France/73 viruses were apparently infected, whereas between 20 and 40 % of mice inoculated with the Italy/72, England/72, Austria/73 and Poland/73 viruses showed signs of infection. The mean dose response curve in mice given this latter group of viruses was extremely flat, ranging from approximately 9 % reactors in mice given $10^{0\cdot5}$ to $10^{1\cdot4}$ p.f.u. to approximately 47 % reactors in mice given $10^{5\cdot5}$ to $10^{6\cdot5}$ p.f.u. The clinical signs shown by affected mice included tremors, hypersensitivity to stimuli, paralysis of one or more limbs and, if death was delayed, considerable wasting. Virus was widely distributed throughout the body, with the greatest amounts of virus being found in the brain (Table 8). Neutralization tests of the sera of some of the mice surviving after 6 weeks confirmed that over 70 % of mice given $10^{0\cdot8}$ to $10^{2\cdot0}$ p.f.u. of six of the seven viruses had suffered inapparent infections (Table 9). The Italy/66 virus proved less infective for this age group and inapparent infections occurred in only four of fourteen mice given $10^{2\cdot9}$ p.f.u.

Table 8. *Distribution of virus in mice dying 7 days after intraperitoneal inoculation of England/72 virus*

Tissue	Infectivity*
Brain	7·36 (6·3–8·2)
Heart	4·10 (3·3–5·1)
Lungs	3·50 (2·8–4·3)
Alimentary tract	4·45 (1·6–5·8)
Carcase	5·00 (2·6–6·4)

* Log_{10} p.f.u./specimen – geometric mean and range of five mice.

Table 9. *The infectivity of SVD virus strains for 5-day-old mice – subclinical infections*

Virus	Inoculum	Number of subclinical infections†/ Number tested
Italy/66	2·9*	4/14
Hong Kong/71	2·0	17/19
Italy/72	1·3	5/15
England/72	1·5	14/15
Austria/73	1·0	15/15
Poland/73	1·4	7/13
France/73	0·8	7/13

* Log_{10} p.f.u.

† Number of mice with neutralizing antibody titres > 2·0 (log reciprocal).

DISCUSSION

These comparative studies have shown that, although there were some differences in the ability of the viruses to produce primary vesicles at the sites of inoculation, six of the seven viruses produced diseases which were similar in severity, distribution of secondary lesions, febrile response and virus excretion. The seventh virus, Italy/66, was considerably less virulent for the pig and many pigs inoculated with virus concentrations up to $10^{5·0}$ p.f.u. failed to acquire infection (Burrows *et al.* 1973). Neutralization tests of convalescent pig sera confirmed that such differences as existed between strains were of a minor degree. The reproducibility of the neutralization test was not good enough to distinguish each virus on the results of a single test but the mean results of three tests confirmed that the Italy/66, Hong Kong/71 and France/73 viruses differed from each other and from the Italy/72, England/72, Austria/73 and Poland/73 viruses. Unequivocal antigenic differences between these four groups of viruses using the same pools of convalescent pig sera have already been demonstrated by agar gel precipitation tests using purified viral antigens (Brown, Talbot & Burrows, 1973) and by complement fixation tests (A. Arrowsmith, unpublished). Differences between the viruses were also seen in the response of 5-day-old mice to intraperitoneal inoculation. The Italy/72, England/72, Austria/72 and Poland/73 group produced signs of disease in a greater proportion of mice than did the other three viruses. The

Italy/66 virus could be differentiated from the Hong Kong/71 and France/73 viruses in that much larger amounts of virus were required to initiate inapparent infections.

The appearance of SVD in widely separated countries after intervals of several years indicates that a reservoir of infection probably exists in some part of the world. Persistence of infection in a semi-immune population over a long period may lead to slight changes in the biological and antigenic properties of the virus. Thus, it was not surprising to find slight differences in virulence and antigenic composition between the Italy/66, Hong Kong/71 and European 1972/73 viruses. The close similarity between the Italy/72, England/72, Austria/73 and Poland/73 viruses suggests that these outbreaks may have had a common origin. A direct link between the Austrian and Polish outbreaks of disease was established (Kubin, 1973) but the original source of these outbreaks or links with other outbreaks has not yet been established.

The France/73 virus differs slightly from the other viruses recovered during 1972/73 and it is possible that the disease in France may not be directly related to the other occurrences of disease in Europe. However, the differences between the experimental and natural diseases produced by the viruses prevalent in Europe in 1972/73 were not sufficient to explain the differences in the spread of disease in the various countries and it is concluded that the apparent differences in epizootiology may be due to other factors relating to the husbandry and marketing of pigs or to the attention paid to what on clinical grounds is a relatively mild disease.

We should like to thank Mrs Jean Huntley and Messrs. G. H. Hutchings and R. Butcher for valuable assistance in the laboratory and in the isolation units, and Messrs. I. Hughes, M. Fortune and M. Tyrell, large animal attendants, for their meticulous attention to handling, cleansing and disinfection procedures. The SVD strains were provided by the World Reference Laboratory of this Institute.

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J. Hyg., Camb. (1974), **73**, 119
Printed in Great Britain

Pertussis agglutinins in vaccinated children: better response with adjuvant

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(*Received* 9 *January* 1974)

SUMMARY

Children were immunized with a single batch of pertussis vaccine, either adsorbed on aluminium hydroxide or plain. With a primary course of three injections, adsorbed vaccine produced higher titres of pertussis agglutinins in the serum than did plain vaccine. There was no obvious difference in response between those who received the three doses at intervals of 1–2 months, starting at 3–4 months of age, and those in whom the third dose was delayed until about 6 months after the second, but the number of children in each group was small.

INTRODUCTION

A previous investigation of pertussis agglutinins in vaccinated children (Abbott, Preston & Mackay, 1971) showed a rather poor response to three doses of plain vaccine given at intervals of 4–6 weeks, starting at 3–4 months of age. The authors recommended a further study to assess the relative efficacies of different immunization schedules and to compare plain vaccine with that containing adjuvant. We are reporting here on the results of such a study.

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MATERIALS AND METHODS

Pertussis vaccine

A single batch of Glaxo vaccine, manufactured in 1970, was used. It was in the form of triple antigen (diphtheria and tetanus toxoids together with 20,000 million cells of *Bordetella pertussis* per 0·5-ml. dose). It had been divided into two portions, one remaining as a plain saline suspension and the other being adsorbed on aluminium hydroxide as adjuvant. Both were stored at 4° C. before the vaccination of children between May 1971 and expiry of the vaccine in October 1972.

Immunization of children

The mothers of children attending the neonatal assessment clinic in Hope Hospital, Salford, were offered a course of injections for their children, to be followed by a laboratory check on the response. The children had no obvious physical disorder, with two exceptions they were not gestationally immature, and they had no known pertussis contact. At the first visit samples of blood were taken from mother and child, and the child received an intramuscular injection of vaccine. Six to ten weeks after the third dose of vaccine, a second sample of blood was taken from the child. On the basis of the laboratory findings, parents were informed of the child's response and, if this was poor, a booster dose was offered.

According to age at the first visit, the children were given a course of injections on either of two schedules: in Schedule I, commonly used in this country for many years, three injections were given at intervals of 1–2 months, starting at 3–4 months of age; in Schedule II, recommended by the Central Health Services Council (1968), the first dose was given at 4–6 months of age, followed by a second dose after about 6 weeks and a third dose about 6 months later. For each schedule, the children were assigned alternately to two groups for immunization with either plain or adsorbed vaccine. At subsequent visits, each child received the same vaccine as previously. In addition, oral polio vaccine was given at each visit; and the Local Authority was notified on the completion of these standard courses of immunization for each child.

Similar conditions applied to children vaccinated in the infant clinic at St Mary's Hospital, Manchester, though a higher proportion was 3–4 months of age at the first visit.

Immunization of rabbits

Ten rabbits were immunized by intravenous injection, five with plain vaccine and five with adsorbed. Each animal, previously shown to have bordetella agglutinin titres of less than 4, received a total of 50,000 million organisms in four doses over a period of 10 days, and was bled again 3 weeks after the last injection.

Four other rabbits were immunized intramuscularly, each with a total of 50,000 million organisms in four doses over a period of 10 days, and they were bled 2 weeks after the last injection. Because of the relatively poor response, they were given a single injection of 20,000 million organisms 2 weeks later, and were bled again after a further 3 weeks. Two months later, they were given a total of 60,000

million organisms in three injections over a period of 7 days, and were bled once more 2 weeks after the last injection.

Estimation of pertussis agglutinins in sera

The content of agglutinin 1 was estimated by titration of serum against the type 1 strain of *Bord. pertussis*, GL353. In the samples which failed to agglutinate this strain the titres of the sera against strains 360E (type 1, 2) and H36 (type 1, 3) were taken to indicate the content of agglutinins 2 and 3 respectively. Sera that contained agglutinin 1 were absorbed with strain GL353 (type 1) until they no longer agglutinated it, and their content of agglutinins 2 and 3 were then estimated by titration of the absorbed serum against strains 360E and H36. The details of these techniques have been recorded by Preston (1966, 1970).

RESULTS

Pertussis agglutinins in normal sera

Table 1 shows that the sera of most of the mothers contained agglutinin 1 but only a minority had agglutinin 2 or 3. Pertussis agglutinin was detected in only nine of the 38 children before vaccination, and the clear relation between the agglutinin titres of the children's sera and those of their mothers' sera suggested that these were residual maternal antibodies. In each case this residual antibody, in children of 3–6 months of age, was only of a low titre.

Agglutinin response to vaccination

Twenty children completed the course of three injections in accordance with Schedule I, and the attendances of eight others could be classed as Schedule II, but the visits of a further five did not conform to either schedule (Table 2). From a study of the combined results with Schedules I and II, it appears that adsorbed vaccine gave a better response than plain. This applied to each of the three pertussis agglutinins but was most significant for agglutinin 2: with adsorbed vaccine all fourteen children achieved a titre of at least 20, but plain vaccine gave a poorer response ($P = 0{\cdot}005$) in which seven out of thirteen children failed to achieve this titre. Schedule II was not obviously better than Schedule I, but the number of children in each group was small. In general, the response to antigen 3 was weaker than that to antigen 2.

The presence of all three pertussis antigens in the vaccine was readily demonstrated in the laboratory by the production of agglutinins in the sera of rabbits injected either intravenously or intramuscularly (Table 3). Perhaps not surprisingly, adjuvant did not enhance agglutinin production after intravenous vaccination. But after intramuscular vaccine, the response in rabbits had similar features to those found in children – a tendency for adsorbed vaccine to give a better response than plain (after injection of 50,000 – 70,000 million organisms), and a weaker response to antigen 3 than to antigen 2. It is worth noting also that the presence of all three antigens, and a suspicion of a lower content of antigen 3, had been detected by the simple procedure of slide-agglutination of the plain

Table 1. *Pertussis agglutinins in normal children and their mothers*

Child		Titres of three pertussis agglutinins in serum of					
		Mother			Child		
Age (months)	Code no.	1	2	3	1	2	3
3	HH2	0	0	0	0	0	0
	SM4	16	10	0	4	0	0
	SM5	32	40	10	4	(10)	0
	HH12	16	0	0	0	0	0
	SM9	4	0	0	0	0	0
	HH9	8	0	0	0	0	0
	SM13	0	0	16	0	0	0
	SM6	32	10	0	8	(10)	0
	SM19	16	0	0	4	0	0
	HH3	32	0	0	0	0	0
3½	SM10	0	0	0	0	0	0
	SM3	16	0	0	(4)	0	0
	SM11	32	0	0	0	0	0
	SM1	16	0	10	0	0	0
	SM7	8	0	10	0	0	0
4	SM8	8	0	0	0	0	0
	SM2	16	0	0	0	0	0
	SM15	4	10	(10)	0	0	0
	SM18	4	0	0	0	0	0
	SM12	32	40	0	0	8	0
	HH14	32	0	0	0	0	0
	SM16	16	0	20	0	0	0
	SM17	(4)	0	0	0	0	0
	SM22	128	20	0	8	0	0
5	SM20	32	20	0	8	0	0
	SM14	0	16	0	0	0	0
6	HH5	8	0	0	0	0	0
	HH6	64	0	0	0	0	0
	HH11	16	0	0	0	0	0
	HH10	16	0	0	0	0	0
	SM21	128	0	0	8	0	0
	SM24	4	0	0	0	0	0
6½	HH1	16	20	0	0	0	0
7	HH13	0	16	0	0	0	0
	SM23	16	0	0	0	0	0
7½	HH4	0	0	0	0	0	0
	HH8	64	10	0	0	0	0
9	HH7	16	0	0	0	0	0

( ), weak reaction.
0, less than 4 (less than 10 for agglutinin 2 or 3 if agglutinin 1 present).

Table 2. *Pertussis agglutinins in sera of vaccinated children*

	Plain vaccine							Adsorbed vaccine						
Course of injections	Child	Ages when injected (months)			Titres of three pertussis agglutinins			Child	Ages when injected (months)			Titres of three pertussis agglutinins		
					1	2	3					1	2	3
Schedule I (intervals of 1–2 months, starting at 3–4 months of age)	HH2	3	4	5	0	0	0	SM5	3	4	5	8	20	0
	SM4	3	4	5	0	0	0	HH12*	3	4	5	8	40	0
	SM10	3½	4½	5½	(8)	10	0	SM9	3	4	5	8	40	10
	SM8	4	5	6	16	10	10	HH9	3	4	5	16	160	40
	SM2	4	5	6	16	80	10	SM13	3	4	5	64	40	10
	SM15	4	5	6	4	80	20	SM3	3½	4½	5½	32	NT	NT
	SM18	4	5	7	32	10	0	SM11	3½	4½	5½	128	160	40
	SM12	4	5½	7	64	320	40	SM1	3½	4½	6	32	320	10
	HH14	4	5½	7½	16	0	0	SM7	3½	5	7	32	80	10
	—	—	—	—	—	—	—	SM16	4	5	6	64	80	20
	—	—	—	—	—	—	—	SM17	4	5	6	128	320	160
Schedule II (later start; delayed third dose)	SM22	4	5½	12½	16	40	0	SM20	5	6½	12½	16	20	20
	HH5	6	7	11	64	160	20	HH10	6	7½	12½	64	40	20
	HH6	6	7	12	64	20	0	SM21	6	7	13	128	160	40
	HH11	6	7½	12½	16	0	0	SM24	6	7½	13½	128	320	40
Other	SM14	5	6½	8	128	320	320	HH1	6½	9	12½	32	160	80
	—	—	—	—	—	—	—	HH4	7½	10	16	64	160	20
	—	—	—	—	—	—	—	HH7†	9	10	21½	32	80	0
	—	—	—	—	—	—	—	HH3‡	10	11	16½	16	160	20

( ), weak reaction.
0, less than 4 (less than 10 for agglutinin 2 or 3 if agglutinin 1 present).
NT, not tested (sample of serum inadequate for absorption).
* three weeks premature.
†, three months premature.
‡, received single dose at 5 months; course re-started at 10 months.

vaccine: agglutination by antibody 1 and by antibody 2 was complete within 3 min. whereas antibody 3 gave complete agglutination within 5 min. but not at 3 min. (For details of technique, see Preston, 1970.)

DISCUSSION

This investigation has confirmed and extended the results of an earlier study with pertussis vaccines manufactured in 1967 (Abbott *et al.* 1971) which showed that three doses of plain vaccine, given to children at intervals of 4–6 weeks, produced a rather poor agglutinin-response. The present findings, with vaccine made in 1970, show a slightly better response even with plain vaccine. But in several cases, agglutinin (especially agglutinin 3) was not detected either in children vaccinated at intervals of 1–2 months, starting at 3–4 months of age, or in those with a later start and a delayed third dose. In the earlier study, a better response was obtained by the injection of a fourth (booster) dose. The present findings show a similar improvement, with only three doses, by the

Table 3. *Pertussis agglutinins in sera of vaccinated rabbits*

Injection			Titres of three pertussis agglutinins					
			Plain vaccine			Adsorbed vaccine		
Route	Total dose of bacteria (millions)	Rabbit	1	2	3	1	2	3
Intravenous	50,000	1	320	1600	800	—	—	—
		2	640	3200	800	—	—	—
		3	320	800	400	—	—	—
		4	320	800	400	—	—	—
		5	320	1600	400	—	—	—
		6	—	—	—	160	800	200
		7	—	—	—	160	800	400
		8	—	—	—	320	1600	400
		9	—	—	—	160	1600	200
		10	—	—	—	160	3200	400
Intramuscular	50,000	11	32	80	10	—	—	—
		12	32	40	< 10	—	—	—
		13	—	—	—	64	160	10
		14	—	—	—	32	320	10
	70,000	11	32	160	40	—	—	—
		12	64	80	10	—	—	—
		13	—	—	—	128	320	80
		14	—	—	—	32	640	80
	130,000	11	256	640	80	—	—	—
		12	512	1280	160	—	—	—
		13	—	—	—	256	320	160
		14	—	—	—	128	1280	320

incorporation of adjuvant in the vaccine. Thus they confirm the conclusions of Feldman (1957) and Butler, Voyce, Burland & Hilton (1969) that adjuvant improves the agglutinin-response to pertussis vaccination, and they show also the extent to which this improvement applies to each of the three major pertussis antigens.

Once again, laboratory tests have been shown to be capable of predicting the agglutinin-response in the child. By agglutinin-production in rabbits, or even by the simple technique of slide-agglutination of the vaccine, it was possible to indicate that all three pertussis antigens were present but that antigen 3 was weaker than antigen 2 in this batch.

Most of the children had no detectable pertussis agglutinin before vaccination (Table 1), but a low titre of agglutinin 1 or 2 or both 1 and 2 was detected in nine of them and was probably residual maternal antibody. However, three of these (SM12, SM20, SM21) gave a good agglutinin response to vaccination (Table 2), and so it seems unlikely that the trace of maternal antibody in three others (SM4, SM5, SM22) was the cause of their poor response. We can provide no evidence, therefore, that residual maternal antibody may seriously interfere with the response to pertussis vaccine, if the course of injections starts at 3 months of age or later.

We wish to record our gratitude to Dr Judith Kleinberg, Dr N. M. Kazi, Sister M. E. Sheard and Sister J. Boucock for their help in the clinical part of this investigation, and to Mr E. J. Carter and Mr F. J. Osman for technical assistance in the laboratory.

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J. Hyg., Camb. (1974), **73**, 127
Printed in Great Britain

Specific immunoglobulin responses in serum and nasal secretions after the administration of attenuated rubella vaccine

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(*Received* 11 *January* 1974)

SUMMARY

The indirect immunofluorescent technique has been used to study the specific immunoglobulin responses in the sera of 63 non-immune adult women who received either Cendehill rubella vaccine subcutaneously, RA27/3 rubella vaccine subcutaneously, or RA27/3 vaccine intranasally. IgG, IgA and IgM antibodies increased virtually simultaneously, starting about 2 weeks after vaccination. IgG antibody appeared in all subjects and reached maximum titres 4–6 weeks after vaccination. The mean IgG titres elicited by the three different methods of vaccination did not differ significantly. IgA and IgM antibodies reached their highest titres between 21 and 28 days after vaccination and then declined to low or undetectable titres within about 9 weeks. The maximum IgA titres observed after intranasal administration of RA27/3 vaccine were significantly higher than those which occurred when the same vaccine was given subcutaneously, but no significant difference in IgM titres was observed. When unfractionated sera were examined IgA antibody was detected in 57 cases (91 %) and IgM in 51 (81 %). Fluorescent examination of fractions obtained by centrifugation on sucrose density gradients frequently revealed small amounts of IgA and IgM antibody which could not be detected by staining unfractionated serum, and with the inclusion of these results IgA antibody was detected in 61 cases (97 %) and IgM in 59 (94 %).

When 39 adults with pre-existing serum antibody were challenged with vaccine a definite IgA response was detected in only one subject and in no case was there any evidence of the appearance of IgM antibody.

Nasal antibody, consisting of IgG or IgA or both, was detected in 17 out of 23 non-immune subjects (74 %) who received RA27/3 vaccine, either subcutaneously or intranasally. Titres were much lower than those which occur in the

natural disease and there was no evidence that nasal antibody was elicited more readily by intranasal than by subcutaneous vaccination.

INTRODUCTION

In acute rubella IgG and IgM antibodies develop rapidly in the blood within a few days of the onset of the rash and reach high titres within two weeks. IgG antibody persists but IgM declines to undetectable titres in a few weeks. IgA antibody also appears in the blood, and secretory IgA appears in the nasal secretions. Bürgin-Wolff, Hernandez & Just (1971) showed that serum IgA antibody reached a peak 5–18 days after the onset of the rash and then declined to low or undetectable titres within three months. Cradock-Watson, Bourne & Vandervelde (1972) and Cradock-Watson, Ridehalgh, Bourne & Vandervelde (1973) also found that IgA antibody followed a transient course, both in the blood and in nasal secretions. However, Ogra *et al.* (1971) found that serum and secretory IgA appeared between one and two months after the rash and persisted for at least a year.

After the administration of attenuated rubella vaccine IgM antibody follows a transient course similar to that which occurs in the natural disease, but the titres attained are lower and IgM may be correspondingly difficult to detect in some cases (Brown & O'Leary, 1970; Ogra *et al.* 1971; Vesikari, Vaheri & Leinikki, 1971; Gupta, Peterson & Murphy, 1972). The appearance of IgA antibody in the serum and nasal secretions after vaccination was studied by Ogra and his colleagues, who compared RA27/3 vaccine given intranasally with HPV77 DK12 vaccine given subcutaneously. After intranasal vaccination the serum and secretory IgA responses were similar to those which had been found to occur in the natural disease, but titres were about twofold lower. After the injection of HPV77 DK12 vaccine IgA antibody was detected in the serum in only 5 out of 30 children but it persisted in these for at least a year; in nasal secretions IgA antibody made only a transient appearance in three cases. Unpublished studies by other workers (quoted by Plotkin, Farquhar & Ogra, 1973) have shown that nasal antibody appears in about 80 % of persons who receive RA27/3 vaccine intranasally and in about 40 % of those who receive the same vaccine subcutaneously, but does not develop after the injection of Cendehill or HPV77 vaccines.

We have used the indirect immunofluorescent technique to study the appearance of specific immunoglobulins in the serum and nasal secretions of adult volunteers receiving attenuated rubella vaccine, either subcutaneously or intranasally, in order to compare the response which follows vaccination with that which occurs in the natural disease and to observe whether the former is affected by the route of administration of the vaccine. In some cases we have centrifuged sera on sucrose density gradients and examined the fractions by immunofluorescence to see if this procedure would improve the detection of low titres of IgA or IgM antibody. This method proved to be very sensitive and we therefore applied it to sera taken after the administration of vaccine to subjects with low initial titres of serum antibody in order to observe whether challenge by vaccine virus was followed by an IgA or IgM response.

MATERIALS AND METHODS

Adults with no pre-existing antibody to rubella

Rubella vaccine was administered to 63 female students, aged 18–22 years, who had no serological evidence of immunity to rubella. Initial haemagglutination-inhibition (HAI) titres were less than 20 in every case and no specific IgG, IgA or IgM antibodies were detected at a dilution of 1/8 by immunofluorescence. These volunteers were divided into three groups for vaccination.

Group 1. 15 volunteers received Cendehill* vaccine subcutaneously in a dose of $10^{3\cdot35}$ TCD 50.

Group 2. 20 volunteers received RA27/3† vaccine subcutaneously.

Group 3. 28 volunteers received RA27/3 vaccine intranasally. The dose of RA27/3 vaccine which was given intranasally was similar to that which was given by injection and was either $10^{3\cdot49}$ or $10^{3\cdot6}$ TCD 50. For intranasal administration the vaccine was reconstituted in 0·5 ml. of distilled water and then dropped slowly into both nostrils while the patient lay supine with the head extended.

Serial blood samples were taken from as many individuals as possible 2, 3, 4 and 6 weeks after vaccination. These specimens were tested for HAI antibody and were examined by the indirect immunofluorescent technique for the presence of IgG, IgA and IgM antibodies. Twenty selected sera taken between 21 and 28 days after vaccination were also centrifuged on sucrose density gradients and the antibody titres in the fractions were measured by the HAI test and by immunofluorescence.

Nasal washings were collected from 9 volunteers in Group 2 and 14 in Group 3 by methods previously described (Cradock-Watson *et al.* 1973). A preliminary washing was taken before the administration of vaccine and from five to eight serial specimens were taken between 1 and 6 weeks after vaccination. Nasal washings were inoculated into cultures of RK13 cells for isolation of rubella virus and were examined by immunofluorescence for the presence of antibody. The total concentrations of IgA were measured by single radial diffusion in commercial immuno-plates,‡ using solutions of 7S IgA as standards.

Adults with pre-existing serum antibody due to natural infection with rubella in the past

In an attempt to discover whether the administration of attenuated virus to persons with pre-existing antibody would be followed by any increase in the titres of immune globulins or by a reappearance of nasal antibody we administered rubella vaccine to 10 male and 13 female volunteers (laboratory staff and students), aged 20–53 years, who already possessed HAI antibody in titres ranging from 20 to 1280 and IgG antibody in titres from 64 to 2048. Five received Cendehill vaccine subcutaneously, 6 received RA27/3 vaccine subcutaneously and 12 received RA27/3 vaccine intranasally. A preliminary nasal washing was taken

* 'Cendevax', manufactured by Smith Kline and French Limited.

† 'Almevax', manufactured by Burroughs Wellcome & Company.

‡ Obtained from Hyland Division of Travenol Laboratories Limited, Thetford, Norfolk.

before the administration of vaccine and serial specimens of serum and nasal washings were taken 2, 3 and 4 weeks afterwards. These specimens were examined by immunofluorescence for the presence of IgG, IgA and IgM antibodies. Nasal washings taken after challenge were also inoculated into RK13 cells for isolation of rubella virus. During the course of this work it appeared that the most sensitive method for detecting small amounts of IgM antibody was to examine density gradient fractions by immunofluorescence; we therefore fractionated sera taken 14, 21 and 28 days after challenge from four of these volunteers whose initial HAI titres were low (20–40) and tested the fractions for specific IgA and IgM as well as for HAI antibody.

We expected that any increase in the titres of immune globulins would be most likely to occur in subjects with low HAI titres. However, only four such volunteers were available among the staff for the prospective collection of serial specimens. We therefore selected, retrospectively, 16 additional patients with low HAI titres (10–80) who had been given rubella vaccine and from each of whom a second specimen of blood had been taken 21–45 days later. Sera taken from these patients after challenge were examined by the HAI test, by immunofluorescence and by centrifugation on sucrose density gradients. Five of these patients had been challenged with Cendehill vaccine, seven with RA27/3 vaccine subcutaneously and four with RA27/3 intranasally.

Immunofluorescent technique

Titres of rubella-specific immunoglobulins were determined by the indirect immunofluorescent technique, using the methods described in our previous work. Cover-slip cultures of BHK21 (clone 13) cells infected with the Judith strain of rubella virus were treated with dilutions of serum, nasal washings, or density gradient fractions and were then stained with fluorescein-conjugated globulins prepared against human IgG, IgA or IgM (Wellcome Reagents Limited). The stained cover-slips were examined in a Reichert microscope, using quartz-halogen dark-ground illumination and an interference exciter filter.

Sucrose density gradient centrifugation

Specimens for centrifugation on sucrose density gradients received prior absorption with chick red cells for at least 1 hr. at 4° C. A volume of 0·5 ml. of a 1/2 dilution of serum, or 0·5 ml. of concentrated nasal washings, was layered on top of a gradient extending from 12·5 to 37·5 % (w/v) which was then centrifuged at 40,000 rev./min. for 17 hr. About 12 fractions were collected after piercing the bottom of the tube. The presence of separate classes of immunoglobulin in serum fractions was detected by double diffusion in agar, using antisera specific for human IgG, IgA and IgM (Wellcome Reagents Limited). The total IgA concentrations in fractions from nasal washings were measured in Hyland immunoplates. Rubella-specific immunoglobulins in the fractions were titrated by the immunofluorescent technique and HAI activity was titrated in microtitre trays.

Table 1. *Serum immunoglobulin responses in 15 adult females after the administration of Cendehill rubella vaccine subcutaneously*

	IgG			IgA			IgM		
Days after vaccination	No. with antibody/no. tested	Range of titres	Median titre	No. with antibody/no. tested	Range of titres	Median titre	No. with antibody/no. tested	Range of titres	Median titre
14	2/13	< 8–64	< 8	4/13	< 8–32	< 8	3/13	< 8–32	< 8
21	14/14	8–1024	181	14/14	16–256	64	13/14	< 8–256	64
28	13/13	8–1024	256	12/13	< 8–256	32	12/13	< 8–128	32
33–45	13/13	16–1024	256	7/13	< 8–128	8	6/13	< 8–32	8
46–53	4/4	128–1024	362	2/4	< 8–32	8	1/4	< 8–16	< 8
54–63	12/12	64–1024	256	*5/12	< 8–32	< 8	†5/12	< 8–32	< 8

* Four months after vaccination IgA antibody was detected in two subjects in titres of 16 and 32, but was no longer detectable in the other three.

† Four months after vaccination IgM antibody was no longer detectable in these five subjects.

Haemagglutination-inhibition titrations

Sera were inactivated at 56° C for ½ hr., absorbed with kaolin, and titrated in WHO plastic trays by the method in routine use in the Manchester Public Health Laboratory (Thompson & Tobin, 1970).

RESULTS

Serum immunoglobulin responses in non-immune subjects

The immunoglobulin responses in Groups 1, 2 and 3 after the administration of three different forms of rubella vaccine are summarized in Tables 1, 2 and 3 respectively. The general pattern of antibody response was similar in the three groups. All three classes of antibody increased virtually simultaneously, starting about 2 weeks after vaccination. IgG antibody appeared in all individuals, increased rapidly in titre during the third week and reached maximum titres 4–6 weeks after vaccination. HAI antibody followed a similar course. The mean final IgG and HAI titres which were attained in the three groups are shown in Table 4 and were four- to eightfold lower than those which develop in the natural disease. The IgG and HAI titres which followed the injection of RA27/3 vaccine were similar to those which followed intranasal administration and appeared to be higher than those elicited by Cendehill vaccine. Differences of this order in the HAI response have been noted in several other studies (see Plotkin *et al.* 1973) but in our groups the differences between the IgG and HAI titres were not statistically significant.

IgA antibody was detected by fluorescent examination of unfractionated sera in 57 subjects (91 %) and IgM in 51 (81 %). In five subjects neither IgA nor IgM was detected. IgA and IgM antibodies increased rapidly in titre during the third week after vaccination and followed similar courses in the three groups. In the majority of individuals the highest IgA and IgM titres were observed about 21 days after vaccination, but in some cases the highest titres occurred a week later and in others the titres at 21 and 28 days were the same. The geometric means of the highest observed titres of specific IgA and IgM are shown in Table 4. The maximum IgA titres elicited by intranasal RA27/3 vaccine were significantly higher than those which were observed when the same vaccine was given subcutaneously ($P < 0{\cdot}05$). The differences between the IgM titres are not statistically significant. After the fourth week IgA and IgM antibodies declined and were detected in only about a third of the subjects 6 weeks after vaccination. Eight or 9 weeks after vaccination further specimens were taken from 28 (45 %) of the subjects, including all those who had shown IgA or IgM antibody at the previous examination. IgA antibody was still present in low titres in nine subjects and low titres of IgM were also present in nine. From the majority of volunteers who still had IgA or IgM antibody at this time further specimens were taken 4 months after vaccination when it was found that IgM antibody had disappeared but that IgA antibody was still present in three cases in titres of 8, 16 and 32.

We sought other evidence for the possible persistence of specific IgA or IgM by staining serum specimens from 43 other women who had been successfully

Table 2. *Serum immunoglobulin responses in* 20 *adult females after the administration of RA*27/3 *rubella vaccine subcutaneously*

Days after vaccination	IgG			IgA			IgM		
	No. with antibody/no. tested	Range of titres	Median titre	No. with antibody/no. tested	Range of titres	Median titre	No. with antibody/no. tested	Range of titres	Median titre
14	2/11	< 8–32	< 8	2/11	< 8–32	< 8	3/11	< 8–16	< 8
21–22	16/17	< 8–2048	128	14/17	< 8–1024	64	14/17	< 8–512	32
25–31	14/14	64–2048	256	10/14	< 8–512	16	8/14	< 8–256	32
33–45	16/16	128–1024	724	5/16	< 8–64	< 8	5/16	< 8–64	< 8
46–53	4/4	128–1024	256	2/4	< 8–64	8	2/4	< 8–32	8
54–63	9/9	256–2048	1024	*3/9	< 8–32	< 8	†4/9	< 8–16	< 8

* Four months after vaccination one subject still had an IgA titre of 8. No further specimens were available from the other two.
† Four months after vaccination IgM antibody was no longer detectable in three subjects. No further specimen was available from the fourth.

Table 3. *Serum immunoglobulin responses in* 28 *adult females after the administration of RA*27/3 *rubella vaccine intranasally*

Days after vaccination	IgG			IgA			IgM		
	No. with antibody/no. tested	Range of titres	Median titre	No. with antibody/no. tested	Range of titres	Median titre	No. with antibody/no. tested	Range of titres	Median titre
14	2/16	< 8–16	< 8	2/16	< 8–32	< 8	3/16	< 8–32	< 8
19–23	25/25	32–2048	256	24/25	< 8–1024	128	21/25	< 8–256	32
25–31	18/18	64–1024	512	15/18	< 8–256	45	13/18	< 8–256	32
33–45	16/16	128–1024	512	6/16	< 8–64	< 8	4/16	< 8–16	< 8
46–53	10/10	128–2048	362	3/10	< 8–32	< 8	3/10	< 8–32	< 8
54–63	7/7	128–2048	1024	*1/7	< 8–8	< 8	0/7	< 8	< 8

* No further specimens were available from this subject.

Table 4. *Geometric mean titres of serum antibodies to rubella in three groups of adults who received different forms of rubella vaccine*

	Group 1 Cendehill vaccine subcutaneously	Group 2 RA27/3 vaccine subcutaneously	Group 3 RA27/3 vaccine intranasally
Mean final HAI titre 42–63 days after vaccination	131	211	188
Mean final IgG titre 42–63 days after vaccination	487	751	724
Mean of highest observed IgA titres*	99	60	141
Mean of highest observed IgM titres*	86	41	50

* Titres of < 8 were regarded as = 4 for the purpose of calculating geometric means.

Table 5. *Rubella antibodies in fractions obtained by centrifuging serum on a sucrose density gradient*

(23 days after intranasal vaccination)

Fraction no.	HAI titre	Immunoglobulin detected by gel diffusion			Immunofluorescent titre of rubella-specific immunoglobulin		
		IgG	IgA	IgM	IgG	IgA	IgM
1	< 2	–	–	–	< 1	< 1	< 1
2	4	–	–	tr	< 1	< 1	4
3	16	–	–	+	< 1	1	16
4	8	–	–	+	< 1	4	4
5	8	tr	–	–	< 1	16	< 1
6	16	+	+	–	16	32	< 1
7	32	+	+	–	16	16	< 1
8	64	+	+	–	64	16	< 1
9	8	+	+	–	16	4	< 1
10	8	+	–	–	2	< 1	< 1
11	4	+	–	–	2	< 1	< 1
12	4	–	–	–	< 1	< 1	< 1
Total units of antibody	.	.	.	.	116	89	24
Titre before fractionation	240	.	.	.	512	256	16

tr = trace.

vaccinated with RA27/3 vaccine 3–6 months previously, either subcutaneously (28 cases) or intranasally (15 cases). Low titres of specific IgA (8–16) were found in three women and an IgM titre of 16 was found in a fourth, but in the remaining 39 cases there was no evidence of persistent IgA or IgM antibody.

In previous work on the specific antibody response in acute rubella it was found that the fluorescent method sometimes failed to stain IgM antibody in dilutions of whole serum but demonstrated it quite clearly in the heavy fractions obtained after centrifuging the same serum on a sucrose density gradient (Cradock-Watson *et al.* 1972). This finding was attributed to competition between specific IgG and

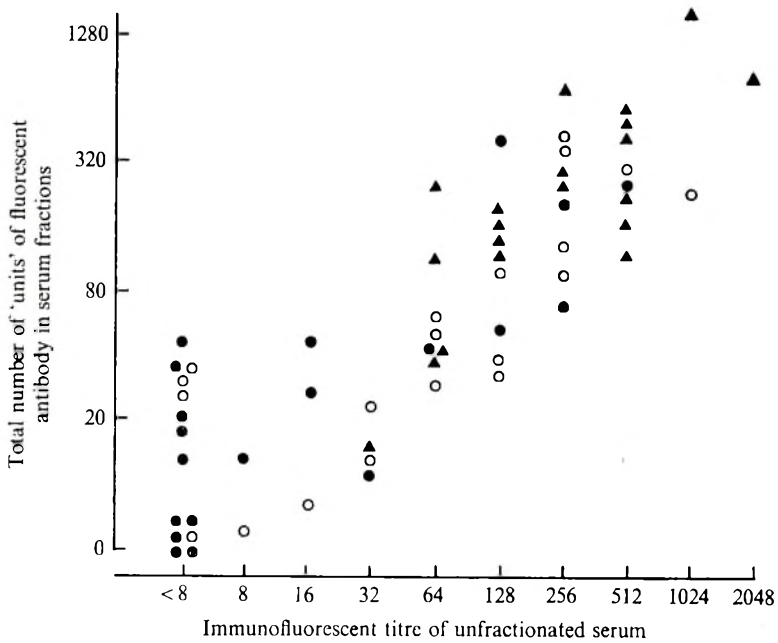

Fig. 1. IgG (▲), IgA (○) and IgM (●) antibody titres of 20 sera taken 21–28 days after the administration of Cendehill vaccine subcutaneously (1 case), RA27/3 vaccine subcutaneously (8 cases) or RA27/3 vaccine intranasally (11 cases). Abscissa = immunofluorescent titre of specific immunoglobulin in unfractionated serum. Ordinate = total number of 'units' of specific immunoglobulin in fractions obtained by centrifugation on a sucrose density gradient (see text).

IgM for the same antigenic sites during the first stage of staining. Because of the possibility that similar competition might affect the detection of IgM antibody in vaccinees we centrifuged a selection of 20 sera taken 21–28 days after vaccination and measured the fluorescent titres of IgG, IgA and IgM antibodies in the fractions. These sera consisted of specimens with a wide range of fluorescent IgM titres and included ten of the twelve sera in which IgM antibody had not been detected when unfractionated material was examined. In each fraction we regarded the reciprocal of the fluorescent titre as indicating the number of arbitrary 'units' of specific antibody. A figure for the total number of units of specific immunoglobulin in a serum was then obtained by adding together the titres in those fractions in which that class of antibody occurred. An example of the results from one serum is shown in Table 5. In Fig. 1 the total number of units of each class of antibody in each serum has been plotted against the fluorescent titre of the unfractionated material. IgG antibody was present in all twenty sera and there was an approximately linear relationship between the fluorescent titre and the total number of units of this antibody in the fractions. In ten sera IgM antibody was not detected when unfractionated material was examined but in eight of these it was found in one or more of the heavy fractions in quantities ranging from 3 to 48 units. In four of these ten sera IgA antibody also appeared to be absent when whole serum was examined but was found in the appropriate fractions in quantities ranging from 2 to 34 units.

Table 6. *Maximum fluorescent titres of rubella antibody in nasal washings from 23 adults who received RA27/3 rubella vaccine subcutaneously or intranasally*

	Number of subjects with the indicated maximum titre of antibody			
	RA27/3 vaccine subcutaneously (9 subjects)		RA27/3 vaccine intranasally (14 subjects)	
Titre	IgG	IgA	IgG	IgA
32	0	0	0	1
16	0	0	0	0
8	0	1	0	0
4	0	2	1	3
2	1	1	3	2
1	4	2	5	2
< 1	4	3	5	6
Median titre	1	1	1	1

In order to assess the relative sensitivity of the HAI test and the immunofluorescent technique for detecting IgM antibody in the heavy fractions we compared the HAI and the fluorescent IgM titres in 45 fractions which contained IgM antibody but not IgG or IgA. In eight fractions the HAI titres were twofold greater than the fluorescent titres and in seven fractions the HAI and fluorescent titres were the same. In the remaining 30 fractions the fluorescent method was more sensitive than the HAI. In 24 of these the fluorescent titres were 2- to 16-fold greater than the HAI titres and in 6 fractions the fluorescent method gave titres ranging from 1 to 4 in the absence of any definite HAI activity.

Nasal immunoglobulin responses in non-immune subjects

Antibody consisting of IgG or IgA, or both, was detected in one or more nasal washings from 17 out of 23 subjects (74%) who received RA27/3 vaccine. IgG antibody was detected between 21 and 42 days after vaccination and IgA between 18 and 36 days, but the two types of antibody were not always present together and were detected erratically in some specimens and not in others. The highest titres which were detected following subcutaneous or intranasal administration are shown in Table 6. An IgA titre of 32 was found in a single nasal washing from one volunteer who had received intranasal vaccine but apart from this instance there was no evidence to suggest that nasal antibody was elicited more readily by intranasal than by subcutaneous vaccination.

The total IgA content of nasal washings ranged from < 3·8 to 10·7 mg./100 ml. In view of these relatively low concentrations it is possible that small amounts of IgA antibody may have escaped detection in some specimens. Low titres of antibody may also have been obscured by nonspecific fluorescence which sometimes occurred when undiluted specimens were stained for IgA. Previous experience with cases of acute rubella, however, had shown that concentrating the specimens

Table 7. *Rubella antibodies in fractions obtained by centrifuging concentrated pooled nasal washings on a sucrose density gradient*

(24 pooled washings from 9 subjects, 21–34 days after intranasal vaccination)

Fraction no.	HAI titre	Immunoglobulin detected by gel diffusion*		Immunofluorescent titre of rubella-specific immunoglobulin	
		IgG	IgA mg./100 ml.	IgG	IgA
1	< 2	–	< 4	< 1	< 1
2	2	–	< 4	< 1	< 1
3	4	–	6·2	< 1	2
4	8	–	10	< 1	8
5	16	–	24	< 1	16
6	8	–	12	< 1	4
7	2	+	4·5	2	< 1
8	2	+	4·5	4	< 1
9	2	–	< 4	< 1	< 1
10	< 2	–	< 4	< 1	< 1
11	< 2	–	< 4	< 1	< 1
12	< 2	–	< 4	< 1	< 1
Titre before fractionation	.	.	44	4	16

* IgG was detected by double diffusion in agar. IgA concentration was measured in Hyland immuno-plates.

aggravated the nonspecific staining and therefore we did not attempt to detect low titres of antibody by concentrating individual washings before testing.

Nasal washings which were found to contain antibody were pooled and the globulins were concentrated by precipitation with half saturated ammonium sulphate. The precipitate from 25 ml. of washings was redissolved in 1 ml. of PBS and 0·5 ml. of this material was centrifuged on a sucrose density gradient. The distribution of rubella antibody in the fractions from pooled washings obtained after intranasal administration of RA27/3 vaccine is shown in Table 7. IgA sedimented more rapidly than IgG and there was no overlap between these antibodies. Although no markers were used the results suggest that nasal IgA antibody was predominantly in the 11S dimeric form and was therefore probably locally produced. When pooled nasal washings obtained after the subcutaneous injection of RA27/3 vaccine were centrifuged the sedimentation pattern was qualitatively similar.

Rubella virus was isolated from the nasal washings of 4 out of 9 persons who received RA27/3 vaccine by injection and 9 out of 14 who received the same vaccine intranasally. Isolations were made between 7 and 25 days after vaccination, from one, two or three specimens per patient. In general, virus tended to appear earlier than antibody in nasal washings but there was some overlap between these events and in four subjects virus and antibody were present in the same specimens. However, there was no definite association between virus isolation

and the presence or subsequent appearance of antibody and in three subjects from whom virus was isolated no antibody was detected.

Responses in subjects with pre-existing serum antibody

Among the 23 staff and students who were challenged with three different forms of rubella vaccine no variation in serum HAI or IgG titres of more than twofold occurred during the period of observation, even in the 4 whose initial HAI titres were low (20–40). Four volunteers had initial serum IgA titres of 8 (1 case), 32 (2 cases) and 64 (1 case) which did not alter after challenge. No serum IgM antibody was detected by fluorescence in any of these subjects at the time of challenge nor did any appear during the four subsequent weeks. Traces of IgG antibody, but not IgA, were detected in one or more nasal washings from five subjects but showed no definite relation to the time of challenge. No virus was isolated from nasal washings from any of these individuals 2, 3 or 4 weeks after the administration of vaccine. There was therefore no evidence of reinfection of the upper respiratory tract in any of these subjects.

From 20 persons with low initial HAI titres (10–80) 28 sera taken between 14 and 45 days after challenge were examined by centrifugation on sucrose density gradients as well as by indirect immunofluorescence in an attempt to detect an IgA or IgM response. Four subjects were from the previously mentioned group of staff and students, and from each of these we examined three sera taken 14, 21 and 28 days after challenge. Sixteen were patients, from each of whom a second specimen of blood had been taken between 21 and 45 days after challenge. Eleven of these patients showed rises of at least fourfold in the titres of HAI or IgG antibody, or both. Six of the twenty subjects had been challenged with Cendehill vaccine (3 rises), eight with RA27/3 subcutaneously (5 rises) and six with RA27/3 intranasally (3 rises). None had any pre-existing IgA or IgM antibody. A definite IgA response occurred in only one case. This was a patient who had been given Cendehill vaccine 35 days previously and who showed a rise in HAI titre from 10 to 120 and in IgG titre from 16 to 32. IgA antibody was detected by fluorescence in the second serum in a titre of 16 and was present in three fractions from this serum in a total quantity of 20 units. It was not detected in fractions from serum taken on the day before vaccination. In one staff volunteer who showed only a twofold rise in HAI titre after subcutaneous challenge with RA27/3, a trace of IgA antibody (2 units) was found 14 and 21 days after challenge, in only a single fraction on each occasion. None was detected in fractions from sera taken before vaccination or 28 days thereafter. However, the significance of such a small amount of IgA is uncertain because it was detected at the threshold of sensitivity of the technique. In no case was there any evidence of an IgM response after challenge with rubella vaccine.

DISCUSSION

The general pattern of serum immunoglobulin response which followed each of the three methods of rubella vaccination was similar to that which we have

found in the natural disease except that titres were about four- to eightfold lower. All three classes of antibody developed simultaneously after a delay of about 14 days which may be regarded as the incubation period of vaccine-induced infection and was the same whether vaccine was given subcutaneously or intranasally.

Serum IgA antibody after vaccination followed a transient course similar to that which we have found in acute rubella. The maximum titres appeared to be significantly higher after intranasal than after subcutaneous administration of RA27/3 vaccine but it is uncertain whether this in fact indicates a greater humoral response. Comparison of the titres attained by temporary antibodies are valid only if samples are taken at peak times and the inevitable failure to do this may tend to enlarge or diminish the differences between groups. Traces of IgA antibody lingered in a few cases but it is probable that this also occurs after the natural disease because we have occasionally detected low titres of IgA in adults who possessed antibody from infection in the distant past.

We detected nasal antibody in the majority of subjects who received RA27/3 vaccine, whether subcutaneously or intranasally, and the sedimentation results suggested that the IgA component was locally produced. The titres of nasal antibody, however, were much lower than those which we obtained by identical methods in acute rubella and in the majority of specimens antibody was detected only in undiluted material. This difference invites a similar comparison between the reinfection rates in the face of natural challenge which are higher among vaccinees than among those with naturally acquired immunity (Hortsmann *et al.* 1970; Davis *et al.* 1971). In previous work, however, we showed that nasal antibody in acute rubella was transient, and in the work described here we found no evidence of recall of nasal IgA when subjects with naturally acquired immunity were challenged with vaccine. In view of these two findings it seems doubtful if the more solid immunity which follows the natural disease can be attributed to greater production of nasal antibody. Immunity probably also depends on cell-mediated mechanisms which in rubella have received very little study.

IgM antibody in vaccinees also followed a transient course similar to that which occurs in the natural disease. Fluorescent staining of unfractionated sera showed persistent IgM in only one case, and although more instances might have been revealed if late sera had been fractionated, persistence of IgM after about 9 weeks is probably uncommon. Live virus occasionally lingers in the body for several weeks after vaccination and might be a risk to the fetus if a woman were to become pregnant during this time, but to what extent persistence of virus is accompanied by prolongation of the IgM response is unknown.

Comparison of fluorescent IgM titres in vaccinees with those obtained in cases of acute rubella is difficult because there is evidence that in sera from cases of natural infection the presence of a high titre of IgG antibody may block the attachment of IgM to antigen during staining and so prevent the detection of quite large amounts of IgM antibody. We have the general impression, however, that considerably less IgM antibody is formed after vaccination and this conclusion agrees with the findings of other workers (Ogra *et al.* 1971; Vesikari *et al.* 1971). Although IgM titres were relatively low we were able to detect this class

of antibody in 51 out of 63 vaccinees by fluorescent staining of unfractionated serum. In eight out of ten sera in which fluorescence had failed to detect IgM we were still able to detect this antibody in the heavy fractions obtained after centrifugation on density gradients. However, the amounts of IgM revealed in this way were small and although blocking may have helped to prevent their detection in whole serum there was no evidence from the results shown in Fig. 1 that blocking was preventing the detection of larger amounts of IgM antibody. Possibly the IgG antibody in vaccinees competes with IgM less avidly than the IgG which develops in higher titre after natural infection. If the cases in which IgA and IgM antibodies were detected only by fluorescent staining of gradient fractions are added to those in which these antibodies were detected by staining whole serum then IgA antibody was detected in 61 cases (97 %) and IgM in 59 (94 %). Allowing for the possibility that sampling may not always have occurred at optimum times it seems reasonable to conclude that both classes of antibody are formed in most, if not all, patients who receive Cendehill or RA27/3 vaccine. In some cases the amounts are likely to be small and will only be detected by the most sensitive methods.

When 39 subjects with pre-existing serum antibody were challenged with vaccine there was no evidence of an IgM response although 20 of these subjects had low initial HAI titres and eleven of these 20 showed fourfold rises in the titres of HAI or IgG antibody, or both. In only one case was a definite IgA response detected. It is possible that abbreviated IgM responses occurred which did not correspond with the times when blood samples were taken but even so it seems likely that IgM responses in patients with pre-existing antibody are rare and that the chances of detecting them are small. Rubella vaccine is occasionally administered inadvertently to women in the early stages of pregnancy whose immune status is unknown (Mair & Buchan, 1972; Wyll & Herrmann, 1973). The detection of IgM antibody in such a case during the fourth week after vaccination would probably indicate that the patient was not previously immune and that there was a risk of fetal infection.

Our results show clearly the value of fluorescent staining of density gradient fractions for detecting low titres of specific IgM. Fluorescent titres were usually higher than HAI titres and fluorescence sometimes revealed traces of IgM antibody in fractions which had no definite HAI activity. When only traces of IgM are present most of the antibody from 0·25 ml. of serum is contained in one or two fractions, each of 0·4 ml., which are thus equivalent to a dilution of about 1/2 or 1/4 of the original serum. In practice the fluorescent detection of such low titres of IgM in whole serum is unreliable and the fluorescent examination of fractions provides a gain in sensitivity because nonspecific staining is less and competition between IgG and IgM cannot occur.

We are grateful to Mr K. M. Thompson, F.I.M.L.T., and his staff for carrying out the HAI titrations, to Mrs Hilary Tillett for statistical help and to the patients and volunteers who agreed to take part in this study. One of us (H.M.) was in receipt of a grant from the Medical Research Council. We thank Dr F. T. Perkins

of the Medical Research Council for the supply of 'Cendevax', and Dr D. S. Freestone of the Wellcome Research Laboratories for the supply of 'Almevax'.

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J. Hyg., Camb. (1974), **73**, 143
Printed in Great Britain

Aetiology and epidemiology of viral croup in Glasgow, 1966–72

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(*Received* 14 *January* 1974)

SUMMARY

A retrospective study of 258 children admitted to Ruchill Hospital, Glasgow, with croup between 1966 and 1972 indicated that the viruses most frequently associated with the syndrome were parainfluenza types 1 and 3 and influenza A. Most cases were admitted in the late autumn and winter months, with a small peak in May and June. This seasonal distribution mirrored the circulation of the main causative agents in the community, parainfluenza 1 being principally associated with the autumn cases, influenza A the winter cases and parainfluenza 3 the summer cases. Two of these 'croup associated' viruses showed regular periodicity, parainfluenza 1 occurring biennially in even years and influenza A in most years. The periodicity of parainfluenza 3 is as yet undetermined.

INTRODUCTION

Viral croup remains an important clinical disease of early childhood which often necessitates admission to hospital and occasionally results in death. Past investigation of the viral aetiology of the disease in North America showed association with parainfluenza viruses of types 1, 2, and 3 (Chanock, 1956; Chanock *et al.* 1958; Beale, McLeod, Stackiw & Rhodes, 1958; Cook *et al.* 1959). Other viruses (influenza A and B; adenovirus types 1, 2, 3, 5 and 7; coxsackievirus A9 and echoviruses 10 and 14) were associated with the syndrome in Melbourne, Australia (Forbes, 1961). More recently the role of respiratory syncytial (RS) virus as a causative agent was reported (Gardner, McQuillin, McGuckin & Ditchburn, 1971). Stark (1969) in his review of croup suggested that the parainfluenza viruses and influenza A and B were the commonest aetiological agents. The present paper reviews the viral aetiology and epidemiology of the cases of croup admitted to Ruchill Hospital in 1966–72.

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Table 1. *Age, sex and virological investigation of croup admissions* – 1966–72

Age – years	Sex M	Sex F	No. tested	No. positive
< 1	35	15	35	16
1	65	18	54	33
2	35	10	33	18
3	16	9	15	7
4	17	4	16	6
5–10	26	4	20	11
11–14	3	1	4	4
Total	197	61	177	95 (54%)

MATERIALS AND METHODS

Clinical records

Records of all croup cases in the 7-year-period notified to the Records Department at Ruchill Hospital were examined; only those with evidence of characteristic vibrant cough and obstructive stridulous respiration were included. The few cases of acute epiglottitis due to *Haemophilus influenzae*, which is also associated with the croup syndrome, were omitted. Of the 258 cases 76 % were male and 52 % were under 2 years of age (Table 1). The severity of illness varied considerably and two deaths occurred. Of those children in whom conventional treatment by humidification of the atmosphere failed to produce sufficiently rapid improvement, fourteen were treated with a corticosteroid, two were intubated and four had tracheotomy. Throat swabs were submitted from 177 (69 %) of the 258 patients for virological investigation.

Virus isolation

Throat swabs were collected in virus transport medium (Grist, Ross, Bell & Stott, 1966) on admission and sent quickly to the Regional Virus Laboratory in the same hospital. Specimens were cultured in rolled monolayers of secondary rhesus monkey kidney and human embryo lung (WI-38 strain) at 33° C, and stationary Bristol HeLa cultures at 36° C. Agents which exhibited haemadsorption or cytopathic effects were identified by conventional methods (Grist *et al.* 1966).

RESULTS

Epidemiology of virologically positive croup cases

The monthly distribution of the 258 cases of croup admitted to Ruchill Hospital between January 1966 and December 1972 is shown in Part A of Fig. 1. Most cases were admitted in late autumn and early winter, 149 (58 %) in the months of October, November, December and January. There was also a small peak in May and June. It is interesting to compare over the 7-year-period these monthly admissions of croup to Ruchill with details in Part B of the monthly isolations of the three principal croup associated viruses in the Glasgow area during that period. Part B gives the total isolations of these viruses at the Regional Virus

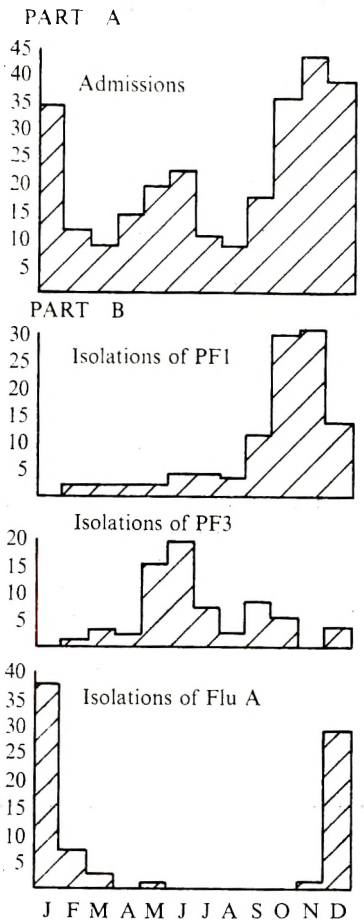

Fig. 1. Monthly distribution in 1966–72 of (A) croup admissions to Ruchill Hospital, (B) isolations from all sources of parainfluenza types 1 and 3 and influenza A viruses.

Laboratory from children under 15 years with a variety of respiratory illnesses in other West of Scotland hospitals as well as Ruchill and also in general practice. It can be seen that the peaks of croup admissions to Ruchill Hospital reflect the peaks of incidence of parainfluenza virus types 1 and 3 and influenza A virus in the community.

Table 2 lists the viruses isolated from 95 (54 %) of the 177 croup patients in Ruchill Hospital from whom swabs were submitted. The viruses isolated in order of frequency were parainfluenza 1 (44), influenza A (18), parainfluenza 3 (14), herpes simplex (7) parainfluenza 2 (3), RS virus (3), adenovirus 1 (2), rhinovirus (2), and one each of adenovirus 5, echovirus 8 and poliovirus 1. In addition two unidentified viruses were isolated. Three of the 95 virologically positive cases were double infections (influenza A and adenovirus 1, influenza A and herpes simplex, and parainfluenza 1 and herpes simplex). During the same period eighteen croup cases (7 %) were associated with clinical measles and three with chickenpox.

Over the 7 years, by far the commonest virus isolated in autumn was para-

Table 2. *Monthly virus isolations from Ruchill croup admissions* 1966–72

	PF 1	PF 2	PF 3	Flu A	R.S.	Herp.	Other
Jan.	—	—	—	10	1	1	Ad. 1 Ad. 5
Feb.	1	—	—	2	—	—	Rh
Mar.	2	—	—	—	—	—	—
Apr.	1	—	—	—	—	1	Rh
May	2	—	3	1	—	—	—
June	2	—	5	—	—	1	—
July	1	—	—	—	—	—	—
Aug.	4	—	—	—	—	—	—
Sept.	5	—	5	—	—	—	—
Oct.	14	—	1	—	—	2	U.T.
Nov.	8	2	—	—	1	2	U.T. A. 1
Dec.	4	1	—	5	1	—	E. 8 P. 1

PF 1, PF 2, PF 3	Parainfluenza Virus Types 1, 2, 3
Flu A	Influenza A Virus
R.S.	Respiratory Syncytial Virus
Herp.	Herpes Simplex Virus
Ad. 1, Ad. 5	Adenovirus Types 1, 5
Rh.	Rhinovirus
E. 8	Echovirus Type 8
P. 1	Poliovirus Type 1
U.T.	Unidentified Agent

influenza 1, although September yielded five isolations of parainfluenza 3 (Table 2). The December cases yielded a mixture of viruses, the commonest being influenza A and parainfluenza 1, while in January influenza A predominated. The summer peak in May and June yielded mainly parainfluenza types 3 and 1. Thus during the periods May–June, October–November and December–January, the croup cases were of different viral aetiology – principally parainfluenza 3, parainfluenza 1 and influenza A respectively.

Epidemiology of the croup-associated viruses

The important 'croup-associated' viruses in the Glasgow area are parainfluenza 1, parainfluenza 3 and influenza A. As previously mentioned Fig. 1 shows the monthly distribution of the seven years' isolations of these viruses from all sources by the Regional Virus Laboratory. Parainfluenza 1 was most prevalent in the months of October–November, coinciding with the maximum admission of croup cases infected with that virus. Similarly the peak of parainfluenza 3 in May–June coincides with the months of maximum admission of parainfluenza 3 croups. December and January were the months in which influenza A was most prevalent in the Glasgow area, again coinciding with the maximum admission of croup cases associated with this virus. Analysis of the diagnoses of cases from which parainfluenza viruses were isolated demonstrates the varying degree of croup-association of parainfluenza viruses which were isolated from children with

Table 3. *Croup-association of parainfluenza viruses isolated at the Regional Virus Laboratory from all sources from children under* 15.

	Parainfluenza 1		Parainfluenza 2		Parainfluenza 3	
	Total	Croup	Total	Croup	Total	Croup
Ruchill Hospital respiratory admissions 1966–72	59	44 (75 %)	5	3 (60 %)	38	14 (37 %)
Sources outside Ruchill Hospital 1966–72	43	20 (47 %)	—	—	27	7 (26 %)
Combined cases by year: 1966	8	1 (13 %)	1	1 (100 %)	2	0 (0 %)
1967	2	1 (50 %)	—	—	6	2 (33 %)
1968	25	15 (60 %)	—	—	7	2 (29 %)
1969	2	0 (0 %)	—	—	29	11 (38 %)
1970	24	21 (88 %)	—	—	11	1 (9 %)
1971	—	—	3	1 (33 %)	7	3 (43 %)
1972	41	27 (65 %)	1	1 (100 %)	3	2 (67 %)
Total 1966–72	102	64 (63 %)	5	3 (60 %)	65	21 (32 %)

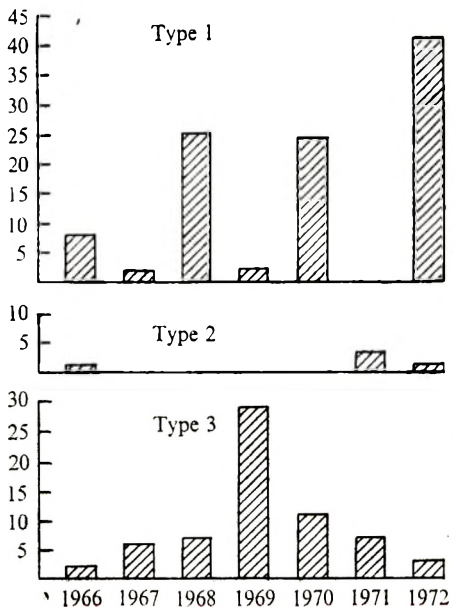

Fig. 2. Parainfluenza isolations 1966–72, Regional Virus Laboratory, Ruchill Hospital.

respiratory infections admitted to Ruchill Hospital and also from swab submissions from all sources outside the Hospital. Table 3 clearly shows an annual variation in the croup-association of the parainfluenza viruses from all sources. Also, although 63 % of the total parainfluenza 1 isolations were croup-associated, only 32 % of total parainfluenza 3 isolations were from croup cases. Parainfluenza 2 was a rare cause of croup in the Glasgow area (three cases) and had a 60 %

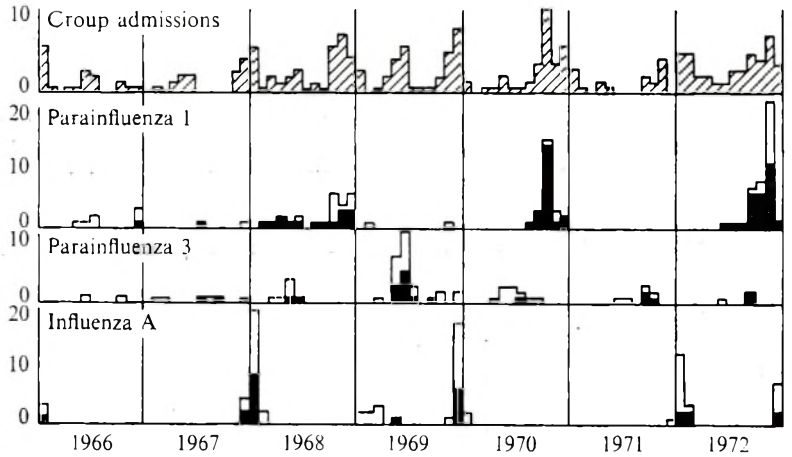

Fig. 3. Monthly croup admissions and total isolations of parainfluenza and influenza viruses 1966–72. ■, isolates from croup cases. □, isolates from other respiratory infections.

croup-association. The 172 isolations of parainfluenza viruses over the 7 year period were from a wide variety of respiratory syndromes in children under 15 years of age. Of this total 70 were submitted from either other hospitals in the West of Scotland (26) or general practice (44).

Annual variations occur in croup admissions and in prevalence of the viruses in the community. Fig. 2 shows the annual isolations of parainfluenza viruses from all sources over the period under investigation. Parainfluenza 1 shows a biennial pattern of raised prevalence in alternate 'even' years, maximal in 1968, 1970 and 1972. Fig. 3 compares over the 7-year period the incidence each month of croup admissions with total isolations of parainfluenza viruses 1 and 3 and influenza A. Thus the only years with peaks of croup in the months of October and November are 1968, 1970 and 1972 with 18, 19 and 16 admissions respectively. During the years of low prevalence of parainfluenza 1, autumnal croup admissions were considerably reduced, e.g. four in 1967. Although there were eight croup admissions in October–November 1971, a year without any evidence of parainfluenza 1 activity, four cases were associated with 'sporadic' viruses (two measles, one each of parainfluenza viruses 2 and 3). Similarly although 1969, another year of low parainfluenza 1 prevalence, had ten late autumn cases, this period with November in particular was marked by RS virus activity. Nevertheless, only one isolation of RS virus was obtained from croup cases during that period. The year 1969 also provides interest by demonstrating an unusually high peak of admissions in early summer which corresponds with the period of maximum isolation of parainfluenza 3 virus during the 7 years. High prevalence of croup in December–January coincides with influenza A activity. Influenza was not detected in the Glasgow area during the winter of 1966-67 when croup admissions were fewer than in any of the other winters (only one in December 1966 and none in January 1967).

DISCUSSION

The dominant importance of parainfluenza and influenza viruses as causes of croup in Glasgow has been shown by the correlation between peak periods of incidence of the disease (reflected in Ruchill Hospital admissions) and peak periods of prevalence of parainfluenza 1, parainfluenza 3 and influenza A viruses as shown by their isolation from general respiratory illnesses as well as croup patients. Unusually low prevalence of these croup-associated viruses correlates with unusually low incidence of croup, as in winter 1966-67.

The maximum number of admissions (43) in the 7-year-period was in the month of November when the total number of virus isolations was not high. Virological studies of other respiratory diseases in the Glasgow area show that this is often a peak month for RS virus activity (Grist, Ross & Stott, 1967; Grist, 1970; Martin *et al.* 1971), and perhaps the high incidence of the disease in November may partly be due to this virus, in keeping with the report from Newcastle that RS virus is an important aetiological agent in croup (Gardner *et al.* 1971).

The outbreak of croup associated with parainfluenza 1 in October 1970, was reflected both by increased admissions of croup cases and also by an increased percentage of the croup-association of parainfluenza 1 isolations during that period. A similar phenomenon was shown by parainfluenza 3 in May–June, 1969. Table 3 shows that the percentage croup-association of parainfluenza infections fluctuates from year to year, tending to be highest when the viruses are more prevalent. Although this might suggest periodic variation in virulence of the viruses, it seems more likely that the figures reflect a sampling bias in favour of hospital admissions, and therefore virological investigation, of children in whom croup is the manifestation of the currently epidemic virus. Thus a raised incidence of croup in any year probably reflects a raised incidence of one of the relevant virus infections in the community, with increased opportunity to infect and help initiate croup in susceptible children. Why some individuals react to the virus in this way remains at the moment speculative, as must consideration of other possible aetiological factors in the croup syndrome.

We are grateful to Dr I. W. Pinkerton, Dr J. H. Lawson and Professor T. Anderson for access to clinical records, and to Dr E. J. Stott, Dr E. J. Bell and Mr B. Cohen for virus isolation data.

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J. Hyg., Camb. (1974), **73**, 151
Printed in Great Britain

A fatal case of cutaneous anthrax

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(*Received* 28 *January* 1974)

SUMMARY

A fatal case of cutaneous anthrax, presenting many unusual features, is reported. No evidence of occupational or domestic exposure to the risk of infection was found.

INTRODUCTION

Anthrax is primarily a world-wide disease of domestic animals affecting cattle, horses, sheep, goats and pigs although infection of many other species has been reported (Christie, 1969). Man is infected through his contact with animals or animal products, person-to-person spread being virtually unknown (Brachman, 1970). Glassman (1958) has estimated the world incidence of human cases to be between 20,000 and 100,000 annually, the vast majority being in countries with a warm climate where pasture land is permanently infected and where the population live in close proximity to domestic animals. Anthrax in humans is rare in this country, eight cases, on average, being reported annually since the disease was made generally notifiable in December 1960. We report here a case of human cutaneous anthrax which was unusual in several respects.

CASE REPORT

The patient was a 68-year-old Irishman, resident for many years in this country. He was in good health until 12 February when he began to feel vaguely unwell. At this time his wife noticed a small swelling below his right ear. During the next 48 hr. the swelling and inflamatory reaction increased and on 14 February he was seen by his family doctor who prescribed lincomycin. However, the swelling progressed and the patient, who was becoming increasingly distressed and anxious, was admitted to an Infectious Diseases Unit on 15 February. On admission he was seen to have extensive subcutaneous oedema extending from the right submandibular area to the anterior chest wall and the left side of the jaw, obliterating the suprasternal notch. On the right side of the neck, over the area of greatest swelling,

there was a well demarcated indurated zone of intense erythema. This area which was slightly elevated and irregular in outline measured approximately 10 cm. by 6 cm. Within this area were several small and one large vesicle, the latter being about 3 cm. in diameter. There was no sign of a developing eschar, nor did one form later. In spite of its appearance, the lesion was remarkably painless. No other abnormality was found, apart from some tachycardia (88/min.) and a temperature of 101° F. Laboratory findings at this time were w.b.c. 6500/mm.^{3} (62 % neutrophils), Hb 17·06 g./100 ml., ESR 4 mm. in 1 hr. (Westergren). A provisional diagnosis of erysipelas was made at this time although it was noted that the oedema was excessive and the lack of tenderness atypical. Three blood cultures, collected at 15 min. intervals, and a swab from the skin lesion were taken before treatment was begun with benzyl penicillin, 2 megaunits six-hourly by intramuscular injection. During the next 24 hr. the patient's condition deteriorated, the oedema extending into the throat and palate and he was unable to speak or swallow. As stridor and respiratory distress were increasing the patient was started on hydrocortisone 100 mg. six-hourly. At this time the results of the three blood cultures taken the previous day were reported as yielding large gram positive aerobic bacilli, provisionally identified as *B. anthracis*. As a result of this report the dose of penicillin was increased to 12 megaunits daily. The immediate concern, however, was for the maintenance of the patient's airway, and the opinion of a consultant in throat surgery was sought. In his hands indirect laryngoscopy revealed marked oedema of the glottis and ulceration of both false and true vocal cords. The surgeon considered a tracheostomy impracticable in the presence of the massive oedema. As it was not possible in the opinion of the anaesthetist to pass an endotracheal tube, a nasopharygeal tube was passed and oxygen administered intermittently in this way, resulting almost immediately in considerable relief to the patient. His condition improved during the next 48 hr., his temperature falling to normal and the oedema subsiding. Results of laboratory tests at this time were w.b.c. 19,900 (neutrophils 75 %) Hb 19·0 g./100 ml., serum urea 240 mg./100 ml. Improvement continued gradually and by 20 February the patient was able to talk a little and to take fluid by mouth. At this time the nasopharyngeal tube was removed and the hydrocortisone was discontinued. However, the white cell count had risen to 38,100/mm.^{3} although all blood cultures (incorporating penicillinase) taken after the onset of treatment did not yield any bacterial growth and a chest X-ray showed clear lung fields.

On the evening of 22 February, one week after admission, at a time when the patient appeared much better, he suddenly became dyspnoeic, collapsed and died within a few minutes. On the instructions of the Coroner an autopsy was not held.

EPIDEMIOLOGICAL INVESTIGATIONS

At work

Mr M. worked in a factory which manufactured cardboard containers. The company appeared to be well run; the buildings were well maintained, the working conditions satisfactory and each worker had his own locker. Since there was only one major manufacturing process the number of raw materials involved was re-

latively small and none was of animal origin. The patient operated a mixing machine which prepared a starch paste, the main adhesive used in the manufacturing process. This paste was made from cornflour, borax, formaldehyde, caustic soda and aluminium sulphate. The cornflour, claimed to be of edible quality, was manufactured in Holland from American yellow maize. It was transported to this country, in sacks by the manufacturers in their own ships which carried no other cargo. In the factory the sacks were stacked near the mixing machine and a forklift truck was provided to lift them. The sacks themselves were made from paper and had not been previously used. Enquiries concerning the shipment of the bulk maize did not reveal any source of contamination with products likely to contain anthrax spores.

Another form of adhesive used on a much smaller scale was stored in another part of the factory. This was a glue made from vegetable materials. However, it came from a factory where other products of animal origin were manufactured and therefore contamination of the vegetable glue or the exterior surface of its container with anthrax spores might have occurred. Apart from this item no other material likely to be exposed to contamination with anthrax spores could be found. There were, however, several piggeries sited close to the factory and these were inspected by district veterinary officers. Evidence of anthrax among the livestock was not found.

At home

The patient lived about one mile from the factory in a small semidetached house in a surburban area. This he shared with his wife, son, daughter and son-in-law. None of these people was in an occupation known to be associated with exposure to anthrax and none had been abroad in the previous 12 months or received gifts from abroad. Mr M. had no pastime or hobby which seemed likely to be relevant, neither had he acquired or borrowed clothing in recent months. There was little family interest in gardening and fertilizer was not found in the house or garden. He travelled to work either by motorscooter or by company minibus through an area where no industrial risk of anthrax was known to the local health authority.

Samples of flour from individual sacks together with flour from the environment and from the patient's boots, were examined by the P.H.L.S. reference expert for anthrax. Further specimens of flour, specimens of the vegetable glue and scrapings from the external surface of its container were examined by us by the method advised by the reference expert. None of the material yielded *B. anthracis*.

DISCUSSION

Three forms of human anthrax, cutaneous, pulmonary and gastro-intestinal are described (Christie, 1969). Pulmonary anthrax is almost invariably fatal but is now exceedingly uncommon, only one case having been reported since 1956 (Dr J. R. H. Berrie, personal communication). The gastro-intestinal form results from eating infected meat and is said not to occur in Britain (Report of the Com-

mittee of Inquiry on anthrax, 1959). The cutaneous form is therefore the only type of anthrax likely to be encountered in this country.

The lesion in cutaneous anthrax arises as the result of the implantation of anthrax spores under the skin. Thus the exposed parts of the body, the face, neck and arms are most frequently affected although statements on how frequently the hand is involved are somewhat contradictory (Christie, 1969; Brachman, 1972). After germinating, the spores multiply locally and produce toxin; in about 5% of cases there is bacteraemia (Brachman, 1970). The severity of cutaneous anthrax varies considerably and the term 'malignant oedema' is used to describe the more severe type of illness characterized by massive progressive oedema, multiple bullae and severe generalized toxaemia (Brachman, 1972). However complete recovery is to be expected in nearly all cases in this form of the disease provided that treatment is prompt and adequate (Report of the Committee of Inquiry on anthrax, 1959).

The case we describe here was bacteraemic and had the features of malignant oedema. Although clinically atypical and with no occupational exposure to anthrax to alert suspicion the provisional diagnosis of erysipelas resulted in prompt, if fortuitous, treatment with adequate doses of the appropriate antibiotic (Christie, 1969; Garrod & O'Grady, 1971). Nevertheless, although the blood was rapidly cleared of the anthrax bacilli, the patient's condition deteriorated for at least a further 24 hr. Indeed the patient's initial response coincided more clearly with the administration of cortisone which has been reported to be of value in malignant oedema (Doust, Sarkarzadeh & Kavoossi, 1968; Tahernia, 1967). However, his progress thereafter appeared satisfactory and his sudden death was totally unexpected.

The severe degree of malignant oedema in this patient presumably reflected the extent of toxin formation. Although prompt antibiotic therapy might be expected to terminate toxin production it would not neutralize the toxin already in the circulation. This would continue to be available to be fixed at the appropriate target sites, explaining the delay in initial response of the patient. It might therefore seem more rational to treat anthrax with antitoxin in addition to antibiotics, and this practice indeed has its advocates and is stated to be the usual procedure in the U.S.S.R. (Lincoln *et al.* 1964; Klein *et al.* 1962). On the other hand Christie (1963, 1969), with considerable experience in treating anthrax, does not think antiserum of value in cases likely to be met in this country and it is evident that antibiotics alone have been effective in most of such cases. Whether the administration of antiserum would further reduce the very small number of fatal cases occurring in this country, is debateable.

The marked polymorpholeucocytosis and haemoconcentration which developed several days before the patient's sudden collapse were perplexing features for which no cause was found. However, it is of interest that these two features are among the pathophysiological changes which develop in rats injected with anthrax toxin (Fish *et al.* 1968).

As an autopsy was not permitted the cause of death in this patient must remain a matter for speculation but it is worthy of note that sudden and unexpected death is a well-documented phenomenon in anthrax (Klein *et al.* 1968; Christie, 1973).

Anthrax in Britain is considered to be almost exclusively an occupational disease, virtually all cases being contracted during the course of industrial, horticultural or agricultural exposure (Report of the Committee of Inquiry on anthrax, 1959). However, in the case reported here we were unable to establish an occupational exposure to materials likely to be contaminated with anthrax spores. Nor was there evidence of domestic exposure, for example, to bone meal fertilizer. This is the only material commonly anthrax-infected to which the general public is likely to be exposed (Jamieson & Green, 1955; Green & Jamieson, 1958; Report of a Working Party of the PHLS, 1959).

The site of the lesion on our patient suggested infection from something carried on the shoulder and we strongly suspected the sacks of cornflour. However, we were unable to isolate the anthrax bacilli from the flour and the patient himself denied carrying the heavy sacks. Furthermore, cornflour is not an animal product and we were unable to obtain a history of any association during shipment or storage with products likely to contain anthrax spores. Such associations of course may be difficult to establish; for example, the cornflour may have been ground in contaminated equipment previously used for grinding bones for bone meal (Dr P. S. Brachman, personal communication).

In the last 3 years two other cases of anthrax have been reported in this country in which the source of infection could not be established. One of these was a foundry worker with fatal meningitis and the second a housewife with cutaneous anthrax who recovered (P.H.L.S. unpublished). Several unexplained cases have also occurred in recent years in the United States (Dr P. S. Brachman, personal communication). Thus while it is most important that occupational exposure should arouse a high index of clinical suspicion of anthrax (Report of the Committee of Inquiry on anthrax, 1959), the absence of such an assocation is no bar to the acquisition of this potentially lethal infection.

We wish to thank Dr Joan Davies, P.H.L.S. Reference Expert for Anthrax, for assistance and advice, Dr Hillas Smith, Neasden Hospital for clinical advice and Mr E. W. Budd, Divisional Public Health Inspector, London Borough of Ealing, for his epidemiological investigations.

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J. Hyg., Camb. (1974), **73**, 157
Printed in Great Britain

Epidemic keratoconjunctivitis in the West of Scotland, 1967–72

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(*Received* 28 *February* 1974)

SUMMARY

Outbreaks of epidemic keratoconjunctivitis have occurred among workers in shipyards and other industrial concerns in the West of Scotland in 1956, 1967 and 1971–72. In the most recent episode 220 persons were known to be affected and those mainly involved were shipyard personnel working on the open decks of ships under construction; only a few non-industrial workers were affected. As in previous outbreaks adenovirus type 8 was shown to be the causal organism. It is likely that spread of the virus was probably facilitated in some of the patients by such procedures as first aid measures to remove foreign bodies from the eye. A survey of the family contacts of those affected in 1971–72 revealed that only 2 % were secondarily infected. This was probably due to propaganda measures to discourage the use of communal face towels, etc.

Despite close virological surveillance over a period of 6 years of patients attending opthalmic clinics in the West of Scotland, there is as yet no clue to the whereabouts of adenovirus type 8 during interepidemic periods. It is suggested that travellers might be responsible for the introduction of the infection into an area.

INTRODUCTION

Keratoconjunctivitis, a disease characterized by unilateral or bilateral inflammation of the conjunctivae, and oedema of the lids and periorbital tissues, and its frequent presentation in an epidemic form, was first described in Austria in 1889 (Fuchs, 1889). Since then many reports of large outbreaks have appeared in the medical literature indicating that the disease has a world-wide distribution

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(Jawetz, 1959). That the disease can cause considerable morbidity was illustrated in 1941 when more than 10,000 cases occurred in Hawaii, at the Naval shipyards of Pearl Harbour and, because of this frequent occurrence of the disease among workers in shipyards, the term 'shipyard eye' was coined. In addition to the descriptions of outbreaks of the disease in shipyards and industrial concerns, several episodes of infection within eye hospitals and clinics have also been described (Wegman, Guinee & Millian, 1970; Barnard, Dean Hart, Marmion & Clarke, 1973). Contaminated instruments and the fingers of medical attendants have often been implicated in such reports.

Keratoconjunctivitis is often a painful and distressing condition causing photophobia, conjunctivitis with severe lacrimation, and discharge. Large follicles may develop on the conjunctiva and corneal opacities may form causing impaired visual acuity often for long periods of time. Although a viral aetiology was suggested in 1930 (Wright, 1930), it was not until 1955 that adenovirus type 8, the most common aetiological agent, was first isolated from a patient (Jawetz *et al.* 1955).

Several outbreaks of epidemic keratoconjunctivitis have occurred in the United Kingdom and at least three – in 1956, 1967 and 1971 – have involved workers in the shipyards and heavy industrial complexes situated in Glasgow and the Clyde Valley (Sommerville, 1958; Taylor, 1967 and Grist, Reid, Bell & Ellis, 1971). The latest outbreak in 1971 presaged three other outbreaks in Britain during the same year – in Bristol in July (Barnard *et al.* 1973), in London and in the Midlands of England in September (Public Health Laboratory Service, 1972).

The activity of adenovirus type 8 in the West of Scotland has been monitored since 1967 during both the epidemic and interepidemic periods to try and study the epidemiological features and reservoir of infection of keratoconjunctivitis. The epidemiological, clinical, and virological findings during this period are reviewed in this paper.

EPIDEMIOLOGICAL FEATURES

Investigation of the outbreaks in 1967 and 1971 was stimulated by reports of cases of keratoconjunctivitis to the Regional Virus Laboratory, Ruchill Hospital, Glasgow, by ophthalmologists and industrial medical officers. Serological tests rapidly established that a member of the adenovirus group was responsible and subsequently type 8 strains were isolated (Bell, Martin & Ross, 1969; Bell, Sneddon & Ellis, 1972).

Outbreaks

The outbreak in 1967 started in August and lasted until December with a peak in September. During that time 382 cases of conjunctivitis were reported from shipyards, engineering and steel works in Glasgow, Clydebank and Motherwell. In one shipyard with 3700 employees 2·2% were affected and in another 4·7% of 2390 employees were involved. Platers, caulkers, drillers, welders and labourers accounted for most of the patients from the shipyards.

Several examples of secondary spread of infection were observed including six virologically confirmed patients with no previous ophthalmic condition or

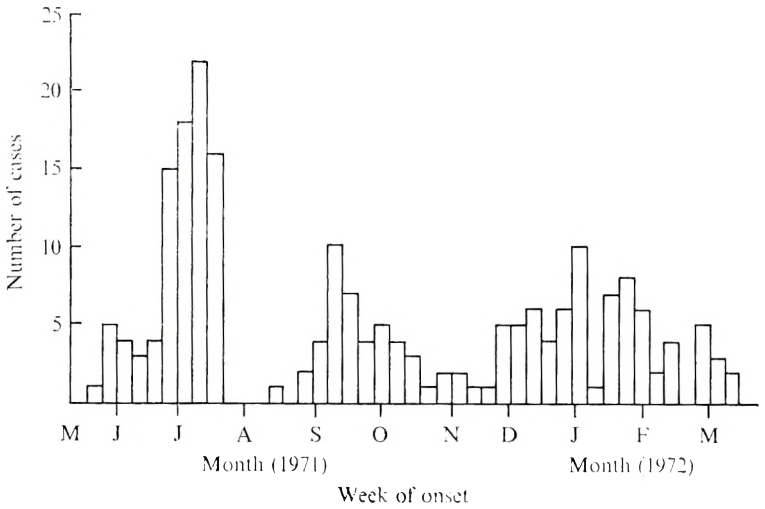

Fig. 1. Number of cases of keratoconjunctivitis occurring in the West of Scotland, May 1971 to March 1972.

industrial contact. They were domestic contacts, within their five families, of hospital-acquired infection in three instances; infection was apparently introduced into the other two families by contact of children with affected playmates. Infection spread to involve all members of two households (two children, both parents and a visiting grandmother in one family; a baby, both parents and a visiting grandmother in the other).

After the cessation of the outbreak in December 1967 very few cases of keratoconjunctivitis occurred until May 1971 when an increasing number of patients with the condition began to be seen at the Eye Infirmary in Glasgow. At first most of the patients came from one of the shipyards in Glasgow which had been involved in the 1967 outbreak and it was only later that workers in other shipyards and industrial concerns in the West of Scotland became infected. The peak of this outbreak was reached at the beginning of July (Fig. 1) The annual vacation taken by industrial concerns in the Glasgow area during the last two weeks of July and first week of August brought a temporary cessation to the number of new cases but, disappointingly, there was a resurgence of infection after the return to work and clinical cases continued to occur until March, 1972.

To try and determine the number of persons involved in the 1971–72 outbreak and collect other epidemiological information, the staffs of ophthalmic clinics and industrial ambulance rooms in the area were asked to collect surveillance data on a standard form which was issued to them. Two hundred and twenty persons presenting with the clinical features of keratoconjunctivitis were recorded.

Of these patients 107 (49%) worked in shipyards (Table 1). The shipyard workers were mostly platers (22%), welders (19%), caulkers (9%), fitters (9%), engineers (7%), electricians (6%), sheet iron workers (6%) and drillers (5%). Among the 113 patients (51%) who did not have a connexion with shipyards, engineers formed the largest group (19% of those affected) followed by clerical workers (8%), housewives (8%) and nurses (6%). Although none of the nurses

Table 1. *Eye infection according to occupation* (1971–72)

Occupation	Number of cases
Shipyard worker	107
Engineer	22
Clerical worker	9
Housewife	9
Nurse	7
Building worker	6
Retail worker	5
School child	5
Other	50
Total	220

Table 2. *Secondary infections in household*

Age group	Affected	Unaffected
< 15 years	4	216
⩾ 15 years	10	368

were employed in shipyards, two worked at clinics in industrial concerns; the remaining five came from local hospitals.

A definite history of injury was obtained from 50 (23 %) of those affected. The remainder presented at the ambulance room or clinic with the features of keratoconjunctivitis as their initial complaint.

Because secondary spread of infection to family contacts was noted in the 1967 outbreak an effort was made in 1971 to gauge the extent of domestic spread. Of 598 family contacts of the 220 patients surveyed, 14 (2 %) were secondarily infected (Table 2). Four of these were under 15 years of age and 10 were older contacts.

Long-term surveillance

An attempt has been made since the 1967 outbreak in Clydeside to accumulate data during the interepidemic period to determine whether the virus smoulders inconspicuously among industrial populations, or among patients attending ophthalmic clinics or whether, as in Japan (Mitsui *et al.* 1955), the reservoir of infection is in children with mild upper respiratory symptoms. To try and solve this problem, virological surveillance of a sample of patients attending ophthalmic clinics with conjunctivitis and keratoconjunctivitis has been undertaken during the past 6 years. From the 957 conjunctival scrapings examined, 203 adenoviruses were isolated, 128 of these being adenovirus type 8 (Table 3). However, in the interepidemic years of 1968, 1969 and 1970, adenovirus type 8 was isolated on only five occasions, all in 1968, and then only from members of a typical industrial group (Grist, Bell & Gardner, 1970).

Table 3. *Virological surveillance of cases of conjunctivitis and keratoconjunctivitis*

	Adenovirus types									
Year	1	2	3	4	7	8	9	10	Not known	Total examined
1967	—	—	11	—	4	57	—	2	1	187
1968	—	—	4	—	3	5	1	—	—	88
1969	—	—	—	—	—	—	—	—	—	35
1970	—	—	6	5	—	—	—	—	—	105
1971	2	1	8	—	8	66	—	1	—	385
1972	3	—	5	1	3	—	2	1	3	157
Total	5	1	34	6	18	128	3	4	4	957

CLINICAL FEATURES

The onset of the illness was acute and heralded by symptoms of irritation often described as a feeling of 'something in the eye', marked lacrimation and photophobia. This was followed by the rapid development of lid oedema, conjunctival infection, chemosis and often a watery discharge. Prominent follicles of the palpebral conjunctiva of both upper and lower lids were usually apparent at the first visit to the clinic (mostly within 4 days of the onset of symptoms). Preauricular adenitis was found in approximately half the cases. In the first week the cornea usually showed a superficial punctate keratitis which developed into very characteristic sub-epithelial infiltrations usually in the second week. There were very few virologically proved cases in which corneal complications did not appear. Follicles persisted for weeks and often months after infection. The sub-epithelial infiltrations were the last signs of keratoconjunctivitis to disappear and in several instances were still present after 18 months. Visual acuity was depressed during the course of the infection but in most cases recovered to the level of vision present before infection, the exceptions being severe cases in which sub-epithelial infiltrations were numerous and persistent.

VIROLOGICAL FINDINGS

Conjunctival scrapings, collected in virus transport medium (Grist, Ross, Bell & Stott, 1966) were examined using the techniques described by Bell and her colleagues (1969). Paired sera were examined by complement fixation and haemagglutination-inhibition tests.

Adenovirus type 8 was the predominant organism in both of the outbreaks (Table 3). During 1967 laboratory evidence of adenovirus type 8 was obtained from 57 (31 %), adenovirus type 3 from 11 (6 %) and adenovirus type 7 from 4 (2 %) of the 187 patients examined. During 1971 evidence of adenovirus type 8 was obtained from 66 (17 %), adenovirus type 7 from 8 (2 %) and adenovirus type 3 from 8 (2 %) of the 385 patients examined.

DISCUSSION

The occurrence of epidemic keratoconjunctivitis in the West of Scotland in 1967 and 1971–72 together with the experience in other parts of the United Kingdom during 1971 illustrates the two principal modes of spread of this potentially preventible disease. The outbreaks in Bristol (Barnard *et al.* 1973), London and the Midlands of England (Public Health Laboratory Service, 1972), were all associated with infection within hospitals and related to ophthalmic investigations and treatment. On the other hand, the outbreaks in Glasgow and the West of Scotland occurred mainly in shipyards and industrial concerns and were not primarily related to the passage of the virus from patient to patient as a side effect of medical techniques. Spread of the disease in both outbreaks mostly occurred outside the hospital or shipyard ambulance room as the majority (77 % in the 1971–72 outbreak) of the patients presented themselves for treatment with the condition and had not previously been to a clinic with another eye disorder. Thus it is likely that spread of the virus within a shipyard is probably due to auto-inoculation or amateur first aid procedures to remove foreign bodies. This is supported by the finding that amongst the shipyard workers the majority of those affected were employed as platers, caulkers and welders, i.e. those workers most likely to receive foreign bodies or flashburns in their eyes. That fingers are an important means of transmission of the virus is also suggested by the fact that ambulance room attendants were affected in the 1967 outbreak and nurses in the 1971–72 outbreak. Infection in ophthalmologists also has been reported by Dawson & Darrell (1963).

In the 1967 outbreak attention was drawn to the spread of the disease within families. This was also noted in the Bristol outbreak in 1971 (Barnard *et al.* 1973). Particular attention was paid to this aspect of the disease during the 1971–72 episode and it was estimated that 2 % of the family contacts developed eye symptoms. It is likely that strong propaganda measures (e.g. the use of separate face towels, etc.) which were instituted at the start of this outbreak had a beneficial effect in limiting the extent of family spread.

A feature of eye infections by adenovirus type 8 is the explosive nature of the epidemics which are, in turn, interspersed with prolonged periods of almost complete absence of the virus. Despite close virological surveillance, over a period of 6 years, of patients attending ophthalmic clinics in Glasgow and the West of Scotland, there is as yet no clue to the whereabouts of adenovirus type 8 during the interepidemic periods, and failure to detect this virus during investigations of acute respiratory illnesses of Glasgow children extending over many years does not suggest a local reservoir of infection in this age group as in Japan (Mitsui *et al.* 1955). However, the fact that outbreaks have occurred in seaports such as Bristol, Glasgow, Copenhagen (Mordhorst & Kjer, 1961) and Singapore (Yin-Coggrave & Loh, 1966) suggests the possibility of the virus being periodically imported from other areas or countries, for example, by seamen, transport drivers, or other travellers.

We should like to thank Mrs L. Steele, Miss B. G. Jack, Mr R. D. Dewar and Dr J. C. M. Sharp, Communicable Diseases (Scotland) Unit, Ruchill Hospital, Glasgow for their assistance with the collection of specimens, analysis of the data and preparation of this paper; Dr C. A. C. Ross and Mrs M. M. Sneddon, Regional Virus Laboratory, Ruchill Hospital, Glasgow for their assistance with the virological investigations; and the large number of hospital and ambulance room personnel who helped with the collection of data.

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Corrigendum

Vol. 72, No. 3; June 1974

On page 478, Table 4, column 1 (Properties) in line 13,
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THE QUARTERLY JOURNAL OF MEDICINE

(NEW SERIES)

CONTENTS

NEW SERIES Vol. 43 No. 170 April 1974
(Vol. 67 of the continuous series)

Price £2.00 net. Subscription price £7.00 per annum

OXFORD UNIVERSITY PRESS

Press Road, Neasden, London NW10 0DD

The Journal of Hygiene Volume 73, No. 1 August 1974

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Printed in Great Britain at the University Printing House, Cambridge