✓Vol. 64, No. 3 September 1966

THE JOURNAL OF HYGIENE

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

CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London, N.W.1

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

Subscription per volume £4. 10*s*. ($17.50) (4 parts)

Single parts 30*s*. 0*d*. ($5.00)

The Quarterly Journal of Medicine

Edited by D. H. BRINTON, W. D. W. BROOKS, R. B. HUNTER, J. D. N. NABARRO, M. L. ROSENHEIM, A. G. W. WHITFIELD

CONTENTS

NEW SERIES, VOL. XXXV NO. 139. JULY 1966
(Vol. LVIII of the continuous series)

Single Numbers 20s. net each Subscription 60s. per annum

OXFORD UNIVERSITY PRESS

Ely House, 37 Dover Street, W. 1

J. Hyg., Camb. (1966), **64**, 255
Printed in Great Britain

The nucleic acid of African horse sickness virus

BY H. MIRCHAMSY AND H. TASLIMI
Razi Serum and Vaccine Institute, P.O.B. 656, Tehran, Iran

(*Received* 27 *February* 1965)

INTRODUCTION

The arboviruses are accepted as a group of RNA-containing viruses which are sensitive to ether and which multiply in and are transmitted by arthropod vectors. African horse sickness virus (AHS virus) is usually classified in this group (Spradbrow, 1964) although its nucleic acid type has not yet been established.

This paper presents some studies on the nature of AHS virus nucleic acid. The effects of actinomycin D on virus growth were examined. This compound combines selectively with DNA, and inhibits DNA-dependent RNA synthesis (Reich, Franklin, Shatkin & Tatum, 1962; Goldberg, Rabinowitz & Reich, 1962).

MATERIALS AND METHODS

Virus

AHS virus mouse-adapted type 2 (OD) and type 6 (S. 114) were used. The stock AHS virus was a pool of 5th to 10th passage virus in MS cells, lyophilized and stored at $-40°$ C. The titres of the AHS viruses were 2 and 6×10^7/ml. plaque-forming units (pfu).

Cells

Line MS of monkey kidney cells was grown in tubes or in 2 oz. bottles for 3–4 days. The growth medium consisted of 5% lactalbumin hydrolysate in Earle's balanced salt solution with the addition of 0·005% yeast extract, 5% inactivated calf serum, and antibiotics. The maintenance medium was the same, but contained only 2·5% calf serum. Adaptation of AHS virus to MS cells has been described in previous reports (Mirchamsy & Taslimi, 1964*b*, 1964*c*).

Drug

Actinomycin D (AD) was kindly supplied by Dr B. Roizman of the Department of Epidemiology, Johns Hopkins University, Baltimore, U.S.A. A stock solution was prepared in sterile 0·85% NaCl at a concentration of 200 μg./ml. This solution was stored in the dark at 0° C. Further dilutions were made in the maintenance medium just before use.

Plaque assay

AHS virus was titrated by a plaque assay method, previously described in detail (Mirchamsy & Taslimi, 1966).

RESULTS

Effect of actinomycin D on the growth of MS cells

Concentrations of 5 μg./ml. or more of actinomycin were toxic for MS cells. The morphological changes were most marked by the second day, when cells had stopped growing. By 24 hr. a noticeable increase in cytoplasmic volume was observed.

The effects of smaller concentrations of actinomycin on cell growth were investigated by counting the number of viable cells in treated and untreated cultures daily for 5 days. The results of a representative experiment are listed in Table 1. Daily observation of actinomycin treated cells indicated that with concentrations up to 0·5 μg./ml. only small foci of cells were granulated. This change became more marked with higher doses, until severe nuclear and cytoplasmic damage or complete cell lysis was observed three days after addition of 2·5–5 μg./ml. of the drug.

Table 1. *Effect of actinomycin D on the growth of MS cells**

Concentration of AD (μg/ml)	Per cent surviving cells after:				
	1 day	2 days	3 days	4 days	5 days
0	95	90	84	81	79
0·05	90	88	80	75	69
0·075	88	85	80	73	68
0·1	85	80	74	70	64
0·25	80	76	73	69	60
0·5	76	71	67	61	54

* Cells from two tubes for each dose of actinomycin D were examined daily. The cells were treated with trypsin, harvested, and then stained with 0·1 % trypan blue.

Effect of actinomycin D on free virus

A suspension of AHS virus type 6 containing 200 pfu/ml. was mixed with 1 ml. of Dulbecco's phosphate buffer saline containing 0·3 μg./ml. of actinomycin, and the mixture was incubated 2 hr. at 36° C. A control sample without drug was included. The samples were then assayed to determine the amount of surviving virus. No change was found under the above treatment.

Virus growth curves

In these experiments, batches of MS cell cultures were infected with AHS virus type 2 or 6 at an approximate multiplicity of 2. After 90 min. at 36° C. to allow adsorption the cultures were washed twice in phosphate buffered saline and re-incubated in 1 ml. of maintenance medium. At 6-hourly intervals pairs of cultures were disrupted by five cycles of freezing and thawing. After centrifugation for 5 min. at 2000 r.p.m. the supernatants were assayed for virus content by the plaque assay method. To determine the effect of actinomycin, batches of MS cells were pre-incubated for 8 hr. with actinomycin D at concentrations of 0·1, 0·25 and 0·5 μg./ml. before addition of virus. The results of a typical experiment are shown in Fig. 1.

In control cultures, AHS virus at a multiplicity of two particles per cell has an eclipse period of 6–8 hr. In cultures treated with 0·1 or 0·25 μg./ml. of actinomycin the eclipse period is much longer, being 12 and 24 hr., respectively, and the final virus yields are much lower than those of the controls. Cultures treated with 0·5 μg./ml. of actinomycin do not seem to produce significant amounts of AHS virus. The inhibition of virus growth by these doses of actinomycin during the first 24 hr. after infection seems to be selective, as no morphological change or significant loss of cell numbers has been observed.

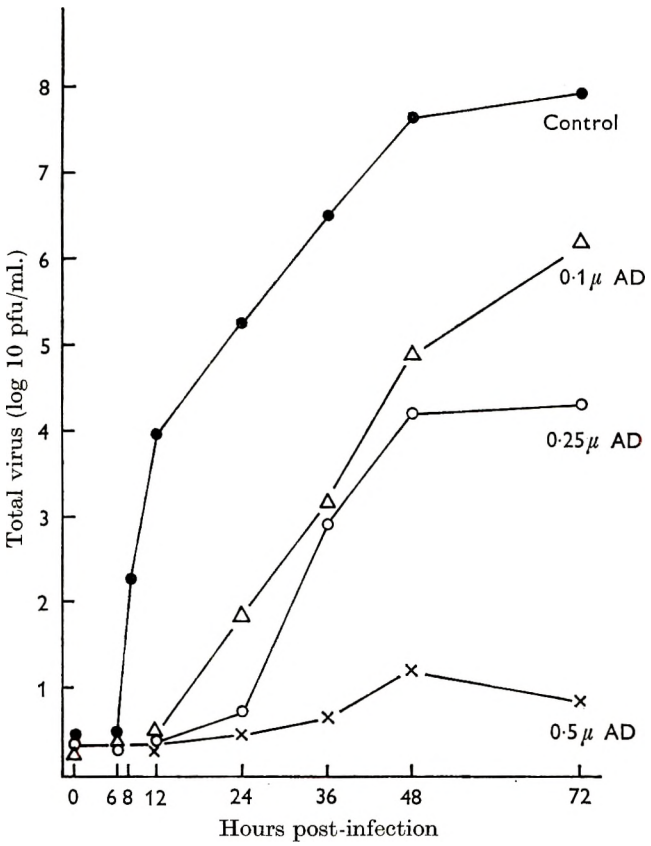


Fig. 1. Growth of African horse sickness virus type 6, strain 114 in MS cells infected at a multiplicity of 2. Cells pretreated with: △, actinomycin D 0·1 μg./ml.; ○, AD 0·25 μg./ml.; ×, AD 0·5 μg./ml.; ●, untreated control.

DISCUSSION

African horse sickness virus is regarded as an arbovirus because of its host range, tissue tropism and the need for vector transmission (Andrewes, 1962). Cooper (1961) has proposed a system of classification based on type of nucleic acid and sensitivity to ether. All arboviruses so far isolated have been found sensitive to ether (Casals, 1961) and to sodium deoxycholate (Theiler, 1957). However, AHS is insensitive to these chemicals (Howell, 1962). Furthermore, there is some doubt whether AHS virus actually multiplies in the insect vector or is

merely transferred mechanically (Ozawa & Nakata, 1965; Mirchamsy & Taslimi, unpublished).

If AHS is not an arbovirus, more information about its chemical make-up is required before it can be classified. Attempts to isolate an infectious nucleic acid by several improved methods of phenol extraction (Wecker, 1959; Di Mayorca *et al.* 1959) have failed (Mirchamsy, Taslimi and Kamali, unpublished data). Consequently, the susceptibility of virus multiplication to actinomycin D was tested, as an indirect means of establishing whether virus nucleic acid synthesis was of the RNA or DNA type.

AHS virus multiplication is inhibited by actinomycin D. This could mean that it is a DNA-containing virus. There are, however, a number of RNA-containing viruses that are also inhibited—influenza virus (Barry, Ives & Cruickshank, 1962), Rous sarcoma virus (Temin, 1963) and visna virus (Thormar, 1965). At least in the case of influenza virus it has been suggested (Barry *et al.* 1962) that virus RNA is made on a DNA template, and that blockage of the template by actinomycin inhibits virus production. On the other hand, the antibiotic may have some effect on the general metabolism of cells which affects some early stage in the growth of susceptible viruses (Temin, 1963). Fluorescent antibody studies (Mirchamsy & Taslimi, 1964*a*) and acridine orange studies (Mirchamsy & Taslimi, unpublished observations) suggest that, like influenza virus, AHS virus multiplies in the nucleus of the infected cell. Since the growth of AHS is sensitive to low concentrations of actinomycin, it is likely that this virus either contains DNA as its genetic material or that there is a stage in virus replication that is dependent on cellular DNA.

SUMMARY

The effect of actinomycin D on the growth of African horse sickness virus in monkey kidney cells was studied. It was found that actinomycin D inhibited the yield of virus. It is suggested that African horse sickness virus is either a DNA containing virus or an RNA virus whose replication is DNA-dependent.

REFERENCES

ANDREWES, C. H. (1962). Classification of viruses of vertebrates. *Adv. Virus Res.* **9**, 271.

BARRY, R. D., IVES, D. R. & CRUICKSHANK, J. G. (1962). Participation of deoxyribonucleic acid in the multiplication of influenza virus. *Nature, Lond.* **194**, 1139.

CASALS, J. (1961). Procedures for identification of arthropod-borne viruses. *Bull. Wld Hlth Org.* **24**, 723.

COOPER, P. D. (1961). A chemical basis for the classification of animal viruses. *Nature, Lond.* **190**, 302.

DI MAYORCA, G. A., EDDY, B. E., STEWART, S. E., HUNTER, W. S., FRIEND, C. & BENDICH, A. (1959). Isolation of infectious deoxyribonucleic acid from S.E. polyoma-infected tissue culture. *Proc. natn. Acad. Sci. U.S.A.* **45**, 1805.

GOLDBERG, I. H., RABINOWITZ, M. & REICH, E. (1962). Basis of actinocymin action, I. DNA binding and inhibition of RNA-polymerase synthetic reactions by actinomycin. *Proc. natn. Acad. Sci. U.S.A.* **48**, 2094.

HOWELL, P. G. (1962). The isolation and identification of further antigenic types of African horse sickness virus. *Onderstepoort J. vet. Res.* **29**, 139.

MIRCHAMSY, H. & TASLIMI, H. (1964*a*). Visualization of horse sickness virus by the fluorescent antibody technique. *Immunology* **7**, 213.

MIRCHAMSY, H. & TASLIMI, H. (1964*b*). Attempts to vaccinate foals with living tissue culture adapted horse sickness virus. *Bull. Off. int. Epizoot.* **62**, 911.

MIRCHAMSY, H. & TASLIMI, H. (1964*c*). Immunization against African horse sickness with tissue-culture-adapted neurotropic viruses. *Br. vet. J.* **120**, 481.

MIRCHAMSY, H. & TASLIMI, H. (1966). The formation of plaques by African horse sickness viruses and factors affecting plaque size. *Can. J. comp. Med.* **30**, 47.

OZAWA, Y. & NAKATA, G. (1965). Experimental transmission of African-horse-sickness by means of mosquitoes. *Am. J. vet. Res.* **26**, 744.

REICH, E., FRANKLIN, R. M., SHATKIN, A. J. & TATUM, E. L. (1962). Action of actinomycin D on animal cells and viruses. *Proc. natn. Acad. Sci. U.S.A.* **48**, 1238.

SPRADBROW, P. D. (1964). Nature and classification of animal viruses. *Aust. vet. J.* **40**, 215.

TEMIN, H. M. (1963). The effect of actinomycin D on growth of Rous sarcoma virus in vitro. *Virology* **20**, 577.

THEILER, M. (1957). Action of sodium deoxycholate on arthropod-borne viruses. *Proc. Soc. exp. Biol. Med.* **96**, 380.

THORMAR, H. (1965). Effect of 5-bromodeoxyuridine and actinomycin D on the growth of visna virus in cell cultures. *Virology* **26**, 36.

WECKER, E. (1959). The extraction of infectious virus nucleic acid with hot phenol. *Virology* **7**, 241.

J. Hyg., Camb. (1966), **64**, 261
Printed in Great Britain

Efficiency of cultures of rectal swabs and faecal specimens in detecting salmonella carriers: correlation with numbers of salmonellas excreted

By CHARLES E. McCALL,* WILLIAM T. MARTIN and JOHN R. BORING

Communicable Disease Center, Public Health Service, U.S. Department of Health, Education and Welfare, Atlanta, Georgia 30333

(*Received* 2 *December* 1965)

The importance of the asymptomatic carrier in the epidemiology of salmonellosis is undenied. Our knowledge of the carrier state itself, however, is grossly inadequate. As yet the carrier site of salmonellas exclusive of *Salmonella typhi* has not been adequately determined, and the patterns of salmonella excretion by asymptomatic carriers are undefined. Carriers are detected primarily by culturing faeces, and it is well known that a single faecal culture frequently may not detect an asymptomatic carrier of salmonellas. Frequently, three consecutive negative faecal cultures are accepted as sufficient evidence that a person is not excreting salmonellas (Mosher, Wheeler, Chant & Hardy, 1941). However, in a recent study at an institution for incurables, it was found that among thirty-six patients who had excreted *S. derby* for 11 months, recovery rates using rectal swab cultures varied widely (McCall *et al.* 1964). Over a third of the carriers could not be detected by culturing three consecutive daily rectal swabs. Speculative reasons for this inefficiency include inadequacy of taking rectal swabs as a method of obtaining faeces for culture, inability of laboratory techniques to detect salmonellas when low numbers are excreted, and intermittent excretion of salmonellas by the carrier.

Before the salmonella carrier state can be intelligently investigated, the efficiency of our methods of detecting carriers must be determined, and if necessary, improved. The excretion patterns of individual carriers also must be defined. The present study was designed to compare the efficiency of culturing rectal swabs and faecal specimens as methods of detecting salmonellas both in carriers of a short duration and in those harbouring the organisms for a prolonged period of time. The culture results are correlated with the excretion patterns of the carriers as depicted by determining the average number of salmonellas excreted per g. of faeces. Additionally, culture results following shipment and a delay in processing were evaluated.

MATERIALS AND METHODS

The subjects used in the investigation were residents at a home for incurables in Philadelphia, Pennsylvania, which was affected by an epidemic of *S. derby*

* Present address: Harvard Medical Unit, Boston City Hospital.

infection. Infections among the residents were first discovered in June 1963. The outbreak within the institution lasted for some 18 months, thus providing the opportunity for studying both recently infected patients and persistent excreters of salmonella. All subjects were asymptomatic carriers at the time of the investigation. They suffered from a variety of chronic diseases, the most common of which were multiple sclerosis, cerebral palsy, Parkinson's syndrome, rheumatoid arthritis, and cerebral vascular disease.

The investigation was divided into two parts. The first part compared the efficiency of culturing rectal swabs and faecal specimens as methods of detecting salmonellas in known carriers, and correlated these results with the number of salmonellas excreted per g. of faeces (wet weight). Two groups of known carriers of salmonella were selected for this part of the investigation.

The first group consisted of six patients who had been negative for salmonella according to the results of four separate rectal swab cultures obtained during the preceding 4 months, but who were positive for the first time at the time of the investigation. Patients in this group were designated as asymptomatic carriers of short duration, and henceforth will be referred to as short-term carriers. Because none of these residents had experienced gastro-intestinal symptoms, the exact duration of infection could not be determined. This group consisted of one male and five females, the ages ranging from 30 to 90 years.

The second group used in the first part of the investigation consisted of eight patients who, 11 months after their initial positive culture, were still positive for salmonella. These eight subjects also had been positive at least once during culture surveys performed at the end of 6-, 7-, and 9-month intervals after their first positive culture. These results suggested that these patients had been chronic carriers since their initial infection. These subjects will be referred to as long-term carriers. There were three males and five females in this group, with ages ranging from 30 to 80 years.

All subjects with the exception of one short-term carrier who excreted *S. anatum*, were carriers of *S. derby*. Rectal swabs and faecal specimens were obtained from both groups. The rectal swabs were taken daily for 11 consecutive days, and faecal specimens were obtained when possible within 6 hr. of the rectal swabs.

The second part of the investigation was designed to determine the effect on culture results of shipment and delayed processing of specimens. In addition to the fourteen subjects in the first part of the study, the group participating in this part included thirty additional 11-month carriers of *S. derby*. Daily rectal swabs were obtained in duplicate for 10 consecutive days from each of these forty-four known salmonella carriers. On the first day, both swabs were processed immediately. Thereafter, one swab was processed immediately and the duplicate swab was held in tetrathionate brilliant green (BG) broth at room temperature for 2 days. It was then shipped air mail from Philadelphia to the Communicable Disease Center, Atlanta, Georgia, for processing. The shipment delayed processing 1 day more.

Bacteriological methods

Rectal swab cultures were obtained using cotton-tipped swabs which were immersed in tetrathionate broth to which brilliant green dye was added to a final concentration of 1/100,000 (weight/volume). The swabs were inserted approximately 5 cm. into the rectum and rotated 3 to 5 times before withdrawing. Faecal specimens were collected in sterile plastic cups, when possible within 6 hr. of the time the rectal swabs were obtained, and were immediately refrigerated.

The same method for isolating salmonella was used for all specimens. Specimens were incubated in tetrathionate BG broth for 24 hr. at 37° C. Samples of the broth were then streaked on brilliant green agar with 8–10 mg. of sulphadiazine added per 100 ml. of agar, and the plates were incubated for 24 hr. Colonies suspected of being salmonella were picked to triple sugar iron agar and the identification confirmed serologically. An average of three colonies per plate were picked.

Estimation of the salmonella content of faecal specimens

The same faecal specimens were used to determine whether the specimen was positive for salmonella and to estimate the number of salmonellas present per g. of faeces (wet weight). To determine the most probable number (MPN) of organisms, a 10 % suspension using between 3 and 10 g. of faeces in tetrathionate BG broth was mixed for approximately 30 sec. in an electric blender, after which serial tenfold dilutions were inoculated in tetrathionate BG broth. Each dilution was made in triplicate. The broth was incubated for 24 hr and streaked on brilliant green agar plates which were then incubated for 24 hr. The presence of salmonellas in each faecal specimen was determined as previously described. When salmonellas were identified, the most probable number per g. of faeces (wet weight) was obtained using standard MPN tables (American Public Health Association, 1960). This method was used in preference to the technique of Miles & Misra (1938) which employs plate counts after dilution. Although we have found the two methods comparable when using pure cultures of *Salmonella in vitro, in vivo*, when the number of salmonellas is low and other types of bacteria are present, determining the MPN using pre-enrichment broth and a selective medium is more sensitive (Boring, unpublished data).

RESULTS

Efficiency of culturing rectal swabs versus faecal specimens, and the excretion patterns of asymptomatic carriers

In Tables 1*a* and *b* the results of cultures of rectal swabs and faecal specimens obtained from short-term salmonella carriers are listed. If the faecal specimen was positive, the number of salmonellas per g. of faeces is recorded. The differences in the ratio of positive cultures to the total number of cultures obtained from each individual ranged from 6/9 to 10/10 for rectal swab cultures, and from 6/7 to 9/9 for cultures of faecal specimens. Of fifty-six rectal swab cultures taken from the six short-term carriers, forty-six (82 %) were positive. Two consecutive rectal swab cultures negative for salmonella were obtained from two subjects. Of thirty-

six faecal specimens collected and cultured over a comparable period, thirty-four (94%) were positive for salmonella and from no patient were cultures of two consecutive faecal specimens negative for salmonella. The average number of salmonellas excreted per g. of faeces per subject ranged between 720 and 61,000. The average for all short-term carriers was 12,000.

Table 1. (*a*) *Culture results of successive daily rectal swabs and faecal specimens from short-term salmonella carriers*

	Subjects											
	1		2		3		4		5		6	
Day	S	F	S	F	S	F	S	F	S	F	S	F
1	+	ND	+	ND	+	ND	+	ND	+	ND	+	ND
2	+	ND	+	ND	−	ND	−	ND	+	ND	−	ND
3	+	+	+	+	+	+	ND	ND	+	ND	+	ND
4	+	+	+	+	+	+	+	ND	+	+	+	+
5	ND	+	ND	+	+	+	+	+	+	ND	+	ND
6	+	+	+	+	+	ND	+	+	+	ND	−	ND
7	−	+	+	+	+	+	+	+	+	ND	−	ND
8	+	+	+	+	−	+	+	ND	+	+	+	+
9	−	+	+	+	ND	−	+	+	+	+	+	ND
10	−	+	−	+	+	+	+	+	+	ND	+	ND
11	ND	−	ND	+	ND	ND	ND	ND	ND	+	ND	ND
Proportion positive	6/9	8/9	8/9	9/9	7/9	6/7	8/9	5/5	10/10	4/4	7/10	2/2

* All subjects excreters of *S. derby* except no. 2 who harboured *S. anatum*.
S, rectal swab; F, faecal specimen; +, positive; −, negative; ND, not done.

(*b*) *Estimated salmonella content of faecal specimens from short-term carriers*

	Subjects					
Day	1	2	3	4	5	6
3	11,000	460	46	ND	ND	ND
4	11,000	4,600	2,100	ND	11,000	46
5	24	4,600	240	11,000	ND	ND
6	240	460	ND	460	ND	ND
7	240	150	240	4,600	ND	ND
8	46	4,600	7	ND	110,000	11,000
9	24	46	0	7,500	11,000	ND
10	46	15	2,400	46	ND	ND
11	0	9	ND	ND	110,000	ND
Average	2,500	1,700	720	3,400	61,000	5,500

* All subjects excreters of *S. derby* except no. 2 who harboured *S. anatum*.

Table 2*a* and *b* list the results of cultures of rectal swabs and faecal specimens obtained from the eight long-term carriers. The ratio of positive rectal swab cultures to total rectal swabs obtained per subject varied from 1/10 to 10/10. One subject had two consecutive negative rectal swab cultures, two had three con-

secutive negative swabs, two had four consecutive negative swabs, two had five consecutive negative swabs, one had eight consecutive negative swabs and one had nine consecutive negative swabs. The ratio of positive cultures of faecal specimens to total number of faecal specimens obtained from each individual varied from 1/3 to 7/7. Only one subject had two consecutive negative cultures of

Table 2. (*a*) *Culture results of successive daily rectal swabs and faecal specimens from long-term salmonella carriers**

	Subject															
	1		2		3		4		5		6		7		8	
Day	S	F	S	F	S	F	S	F	S	F	S	F	S	F	S	F
1	+	ND	+	ND	+	ND	−	ND	+	ND	+	ND	+	ND	+	ND
2	+	ND	−	ND	+	ND	−	ND	+	ND	−	ND	+	ND	−	ND
3	−	ND	−	ND	+	+	−	ND	+	ND	−	ND	+	ND	−	ND
4	+	+	+	+	+	ND	+	ND	+	+	−	ND	+	+	−	+
5	+	+	+	+	+	+	+	+	+	ND	−	−	+	+	−	ND
6	+	+	+	ND	+	+	+	+	+	+	−	ND	+	+	−	+
7	−	+	+	+	+	+	−	ND	+	+	−	ND	+	ND	−	ND
8	−	+	−	+	+	+	−	ND	+	ND	−	−	+	ND	−	ND
9	−	+	−	+	+	ND	−	−	+	ND	−	ND	+	ND	−	ND
10	−	+	−	+	+	ND	−	+	+	+	−	+	+	ND	+	+
Proportion positive	5/10	7/7	5/10	6/6	10/10	5/5	3/10	3/4	10/10	4/4	1/10	1/3	10/10	3/3	2/10	3/3

* All subjects excreters of *S. derby*.
S, rectal swab; F, faecal specimen; +, positive; −, negative; ND, not done.

(*b*) *Estimated salmonella content of faecal specimens from long-term carriers*

	Subjects							
Day	1	2	3	4	5	6	7	8
3	ND	ND	1,100	ND	ND	ND	ND	ND
4	11,000	460	ND	ND	1,100	ND	240	24
5	11,000	1,100	1,200	1,100	ND	0	240	ND
6	1,100	ND	460	39	24	ND	24	3
7	3	9	240	ND	9	ND	ND	ND
8	24	1,100	11,000	ND	ND	0	ND	ND
9	110	240	ND	0	ND	ND	ND	ND
10	11,000	460	ND	46	100	240	ND	4
Average	4,900	530	6,900	300	310	80	170	10

* All subjects excreters of *S. derby*.

faecal specimens. The total ratio of positive rectal swab cultures to the total number of rectal swabs taken was 46/80 (58 %), whereas, the total ratio of positive cultures of faecal specimens to the total number of faecal specimens obtained was 32/35 (91 %). The average number of salmonellas excreted per g. of faeces per subject varied between 10 and 6900. The average for all long-term carriers was 1700.

The comparative results of cultures of rectal swabs and faecal specimens collected from the six short-term carriers and the eight long-term carriers are summarized in Table 3.

In an effort to correlate the proportion of positive rectal swab cultures with the number of salmonellas excreted per g. of faeces, the number of faecal specimens with a given quantity of salmonellas was compared with the results of the rectal swab cultures obtained within 6 hr. of the time the faecal specimen was collected. The correlations appear in Table 4. When fewer than 100 organisms were

Table 3. *Comparison of rectal swabs and faecal specimens collected from short-term and long-term salmonella carriers*

	Number collected	Number positive	Per cent positive
Rectal swabs, short-term carriers*	56	46	82
Rectal swabs, long-term carriers†	80	46	58
Faecal specimens, short-term carriers	36	34	94
Faecal specimens, long-term carriers	35	32	91

* Represents carriers excreting salmonella for less than 4 months.
† Represents carriers excreting salmonella for 11 months.

Table 4. *Correlation of positive rectal swab cultures with number of salmonellas excreted per g of faeces**

No. of salmonellas / g of faeces (wet weight)	No. rectal swabs positive / total no. rectal swabs	Per cent positive
0	0/3	0
10	3/6	50
10–99	7/14	50
100–999	13/17	76
1,000–9,999	9/10	90
10,000–99,999	11/12	92
> 100,000	1/1	100

* Rectal swabs and faecal specimens collected within a 6 hr period on the same day.

present, 50 % or less of the rectal swab cultures were positive whereas twenty-one of twenty-three rectal swab cultures were positive when greater than 1000 salmonellas per g. of faeces were present. No rectal swab culture was positive when a faecal specimen was negative. In contrast, nineteen cultures of faecal specimens yielded salmonella when rectal swab cultures were negative.

Effect of shipment and delayed processing on recovery rates

No significant effect on recovery rates was noted when rectal swabs were shipped and processing was delayed for a period of 1 to 3 days. The results of shipment and delayed processing are listed in Table 5. Of the 440 rectal swabs processed immediately, 230 were positive. Of the remaining 440 rectal swab specimens simultaneously collected and processed after a 1, 2, or 3-day delay, 226 were positive.

Although no significant difference was noted in the total number of positive cultures obtained, in some cases only one of the two swabs from a patient collected simultaneously was positive. By processing both rectal swabs, one immediately and one following a 1-, 2-, or 3-day delay, twenty-one positive cultures were obtained which would have been missed if only one swab had been processed. Two swabs collected simultaneously, therefore, increased the efficiency of rectal swab cultures in detecting carriers by 9 %.

Table 5. *Effect of shipment and delayed processing on the recovery of salmonellas from known excreters*

Time of culture*	No. positive	No. positive in control
No delay	20	21
1-day delay	72	72
2-day delay	67	67
3-day delay	67	70
Total	226	230

* Includes shipment delay of 1 day.

DISCUSSION

In 1955, Thomson, using the quantitative technique of Miles & Misra (1938), studied the number of pathogenic bacilli excreted in the faeces of individuals suffering from acute bacterial gastroenteritis caused by *Shigella*, enteropathogenic *Escherichia coli*, and *Salmonella*. Most subjects with acute salmonella gastroenteritis excreted greater than 1×10^6 pathogens per g. of faeces (wet weight). In the same study, Thomson found that in asymptomatic excreters, the number of salmonellas excreted per g. of faeces tended to be slightly less. Of the six cases of asymptomatic salmonella infection studied, the numbers of salmonellas per g. of faeces were 5×10^6 to 5×10^7 in one case, 1×10^5 in three cases, and 1×10^4 in two cases. No mention was made of how long the patients had been excreting salmonellas, but they would seem to fit our category of short-term carriers. Because of the insensitivity of the Miles and Misra technique when estimating the salmonella content of faeces in mixed culture, individuals harbouring less than 10,000 salmonellas per g. of faeces might have been missed. By utilizing pre-enrichment broth and selective media, low numbers of salmonellas can be detected and estimated by determining the MPN.

The results of this investigation indicate that the number of salmonellas per g. of faeces excreted by short-term carriers and by long-term carriers varies from day to day. The variations result in low numbers of salmonellas being excreted at times. Indeed, at times, salmonellas are either absent or cannot be detected by present bacteriological methods. That the low yield of positive rectal swab cultures obtained from known excreters was, at least in part, related to the low number of salmonellas sometimes excreted by these carriers, was indicated by the comparison between positive rectal swab cultures and quantitative faecal analysis. When

fewer than 1000 salmonellas per g. of faeces were present, recoverability of salmonellas by culturing swabs was poor.

Cruickshank & Swyer (1940), using MacConkey medium, demonstrated in symptomatic hospital patients that cultures of rectal swabs were superior to cultures of faecal specimens in detecting shigellas. Shaughnessy, Frierer & Snyder (1948) in a study of chronic salmonella carriers suggested that catharsis improved recovery rates, but there were no significant differences in the rates of recovery from rectal swabs and faecal specimens. In the present study, the superiority of culturing a faecal specimen of 3–10 g. over culturing a rectal swab was clearly demonstrated. Although the difference was greater in the long-term carriers, it was also significant in the short-term carriers. The higher yield of positive cultures in short-term carriers probably resulted from a greater number of salmonellas per g. being excreted, as was suggested by an average of 12,000 organisms per g. of faeces recovered from short-term carriers as opposed to 1700 per g. of faeces recovered from long-term carriers.

Although the rectal swab culture is valuable for determining the presence of salmonellas in large culture surveys, it cannot be relied upon to detect carriers unless several swabs are taken, the number depending upon the duration of the carrier state and the number of organisms excreted. In this study, three consecutive rectal swab cultures were needed to detect all six short-term carriers, and nine consecutive rectal swab cultures were needed to detect the eight long-term carriers of salmonellas. Two consecutive cultures of faecal samples would have detected all six short-term carriers and would have missed only one of the eight long-term carriers.

Although the second part of the study revealed that by collecting two rectal swabs simultaneously, the efficiency of rectal swab cultures in detecting salmonella carriers could be improved by approximately 9%, the results would still be inferior to culturing 3–10 g. of faeces.

Delayed processing and shipment of rectal swab specimens had no deleterious effect upon the total number of positive cultures obtained from the salmonella carriers. This is comforting, in that cultures frequently have to be shipped for analysis, thereby resulting in a processing delay of several days. Studies are needed to determine whether such delays in processing might affect the result obtained by culturing faecal specimens instead of rectal swabs, and whether cultures of faecal specimens are superior to rectal swab cultures in identifying symptomatic cases of salmonellosis.

SUMMARY

The comparative efficiency of cultures of rectal swabs and faecal specimens in detecting salmonellas in asymptomatic carriers was determined and then correlated with the number of salmonellas excreted per g. of faeces (wet weight). In six short-term salmonella carriers, 82% of rectal swab cultures taken daily for 11 consecutive days were positive for salmonellas, whereas 94% of faecal specimens cultured were positive. Similarily, in 8 long-term carriers, 58% of the rectal swab cultures were positive as compared with 91% of faecal specimens. The

inferiority of the results of rectal swab cultures correlated with their inefficiency in detecting salmonellas when fewer than 1000 salmonellas per g. of faeces were present.

The patterns of salmonella excretion as depicted by estimated salmonella content of faecal specimens showed that all carriers had irregular day-to-day variations. The average number of salmonellas excreted per g. of faeces by short-term carriers was greater than that by long-term carriers, although both groups revealed the same wide variations in number of organisms excreted.

The effect of delayed processing and shipment of specimens upon recovery of salmonellas was also studied. No deleterious effects were noted when specimens were shipped and processing was delayed for up to 3 days.

The authors are indebted to Mary Nelson, Jean Thompson, and Alberta Bekeris for their technical assistance. Acknowledgement is also given to Dr Glenn Smith, Dr Marian Wickingsson, Theodore Last, Teresa Wilson, and the entire nursing staff at Inglis House, Philadelphia, for their cooperation and assistance in this study, and to Jim Goldsby for the statistical analyses.

REFERENCES

American Public Health Association, American Water Works Association, Water Pollution Control Federation (1960). *Standard Methods for the Examination of Water and Waste Water*. American Public Health Association, Inc., pp. 494–507.

Cruickshank, R. & Swyer, R. (1940). An outbreak of Sonne dysentery. *Lancet* ii, 803.

McCall, C. E., Sanders, W. E., Boring, J. R., Brachman, P. S. & Wickingsson, M. (1964). Delineation of chronic carriers of *Salmonella derby* within an institution for incurables. *Antimicrob. Agents Chemother.* **4**, 717.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

Mosher, W. E., Wheeler, S. M., Chant, H. L. & Hardy, A. V. (1941). Studies of the acute diarrhoeal diseases. V. An outbreak due to *Salmonella typhi murium*. *Publ. Hlth Rep., Wash.* **56**, 2415.

Shaughnessy, H. T., Frierer, F. & Snyder, A. (1948). Comparative efficiency of rectal swabs and fecal specimens in detecting typhoid and salmonella cases and carriers. *Am. J. publ. Hlth.*, **38**, 670.

Thomson, S. (1955). The numbers of pathogenic bacilli in faeces in intestinal diseases. *J. Hyg., Camb.* **53**, 217.

J. Hyg., Camb. (1966), **64**, 271
Printed in Great Britain

Salmonellas of subgenus III (Arizona) isolated from abattoirs in England and Wales

By R. W. S. HARVEY and T. H. PRICE
Public Health Laboratory, Cardiff
and J. M. S. DIXON
Public Health Laboratory, Ipswich

(*Received* 14 *March* 1966)

Salmonellas that belong to subgenus III, often termed Arizona strains, have attracted little attention in the United Kingdom, although these organisms can sometimes produce severe and fatal infections in man. The most likely reason for this is the rarity of isolation of these organisms.

A human infection with a subgenus III strain has, however, been recorded in Europe under conditions in which there was little doubt as to the pathogenicity of the organism (Edwards, Kauffmann & Stucki, 1960). This infection occurred in a man returning from holiday in the Balearic Islands.

Late-lactose-fermenting strains of subgenus III may easily be mistaken for salmonellas of sub-genus I. Antigenic overlapping between subgenus III and subgenus I is common with both O and H antigens. The H antigens may be diphasic or monophasic and there is a tendency to regard diphasic varieties as relatively non-pathogenic to man. Rapid lactose fermentation is often recorded in diphasic strains.

During an investigation into the salmonella content of Indian crushed bone several subgenus III strains were isolated (Harvey & Price, 1962). All were diphasic and required 40–64 hr. to ferment 1% lactose peptone water. On brilliant green MacConkey agar (Harvey, 1956), however, some serotypes produced apparently non-lactose fermenting colonies, some were frank lactose fermenters and some produced a mixture of both lactose-fermenting and non-lactose-fermenting colonies, which were sharply differentiated one from the other. One serotype found had previously been encountered in Indian monkeys imported into Germany, Canada and America (P. R. Edwards, personal communication).

Strains of subgenus III were noticeably absent from a recently published list of salmonellas isolated from human, animal and other sources and identified in Great Britain between 1951 and 1963 (Taylor *et al.* 1965). The few isolations recorded here may therefore be of interest.

MATERIALS

The observations on Indian bone, at one time an ingredient of animal feeding stuffs distributed in South Wales, prompted us to examine native animal material. Because the incidence of subgenus III salmonellas was expected to be very low, a

pooling procedure had to be used (Harvey, 1957; Newell, 1959). Four abattoirs were examined using gauze swabs placed in drains receiving material from cattle, pig and sheep slaughter. These swabs were selectively cultured with a view to isolating salmonellas (Moore, 1948; Harvey & Phillips, 1961). Two of the abattoirs (Cardiff and Barry) were in South Wales and two were in England (Beccles I and Beccles II). Some details of the proportions of animals killed in these slaughter houses have already been published (Report, 1964).

METHODS

The techniques used in both laboratories for the isolation of subgenus III strains of salmonellas were very similar. Selenite F broth was used as enrichment medium for culturing the Moore's swabs. Enrichment broths were incubated at 37 or 43° C. or sometimes at both temperatures (Harvey & Thomson, 1953). Subcultures were made at various times from 24–72 hr. on de Loureiro's (1942) modification of Wilson and Blair's medium, on which strains of subgenus III formed characteristic black colonies with surrounding sheen. Other selective plating media were occasionally used successfully, but Wilson and Blair's medium was found to be the most satisfactory. Plates were examined after incubation at 37° C. for 24 and 48 hr. Suspicious colonies were picked and examined for the biochemical properties of members of the genus *Salmonella*. The antigenic structure of the strains isolated was kindly determined by Dr P. R. Edwards and by Dr Joan Taylor.

Table 1. *Details of abattoirs observed*

Abattoir	County	Animals killed in month of isolation of strain: Pigs	Cattle	Sheep	Year and month of isolation	Total period of observation
Cardiff	Glamorgan	2386	836	2757	1965 Apr.	1957–65
Barry	Glamorgan	323	276	1877	1961 Jan.	1960–65
Beccles I	Suffolk	82	684	279	1962 Feb.	1961–62
Beccles II	Suffolk	65	27	17	1962 Feb.	1961–62

Table 2. *Isolations of subgenus I and subgenus III from abattoirs*

Abattoir	Total swabs examined	Swabs positive for subgenus I	Isolations of subgenus III: Swabs positive	Class of animal sampled	Serotype of subgenus III
Cardiff	880	347 (39)	3 (0·3)	Cattle + sheep	26:29–30
Barry	434	122 (28)	1 (0·2)	Cattle + pigs + sheep	26:29–30
Beccles I	150	110 (73)	1 (0·7)	Cattle + pigs + sheep	26:30
Beccles II	177	34 (22)	1 (0·6)	Cattle + pigs + sheep	26:30
Total	1641	613 (37)	6 (0·4)		

Figures in brackets are percentages.

RESULTS

Details of the four abattoirs from which subgenus III strains were isolated are given in Table 1.

Details of the isolation of subgenus III strains are given in Table 2. Four of the strains were diphasic and two were monophasic.

No strains of subgenus II were encountered in these four abattoirs. The six swabs, from which salmonellas of subgenus III were cultured, sampled drains receiving material from the slaughter of cattle, pigs and sheep, or of *cattle and sheep only*.

DISCUSSION

It must be emphasized that very few isolations of subgenus III salmonellae were made. In a period of 9 years in which 1641 abattoir swabs were examined only six were found positive. The only point common to the six positive samples was that the drains concerned received material from cattle and from sheep. Cattle are presumed to be one of the main sources of salmonella in British abattoirs. Sheep are regarded as being relatively infrequently infected (Report, 1964). It was, therefore, natural to think that the subgenus III salmonellas might have a bovine origin. Reference to the Communicable Diseases Centre, Atlanta, Georgia, however, showed that serotype 26:29–30 (isolated four times) was most commonly found in sheep. Of thirty-five strains identified by the Communicable Diseases Centre to 30 June 1965, twenty-three came from sheep and four from other animals. It was interesting to note that on three occasions serotype 26:29–30 was cultured from man and, in two of these incidents, those infected belonged to sheep-herding tribes of American Indians (W. H. Ewing, personal communication). No useful information was available on the monophasic serotype.

Culturally all the abattoir strains of subgenus III appeared as non-lactose fermenting colonies when plated on lactose-containing selective media. In fluid media (1% lactose peptone water), however, the diphasic strains fermented lactose in 48–52 hr, while the monophasic varieties failed to ferment lactose in 7 days. We confirm that the most useful differential medium for subgenus III salmonellas is bismuth sulphite agar, on which, in our experience, the colonial appearance is consistent.

Lastly, this investigation demonstrates the sensitivity of the Moore's swab as an instrument of survey. It is very probable that without this technique the presence of subgenus III salmonellas in native abattoirs could not have been demonstrated.

SUMMARY

Using Moore's swabs in four abattoirs, six strains of subgenus III salmonellas were isolated—four diphasic strains belonging to one serotype and two monophasic strains belonging to another. The serotypes differed in the length of time needed to ferment lactose. There was some evidence that the diphasic serotype could have had an ovine origin.

REFERENCES

EDWARDS, P. R., KAUFFMANN, F. & STUCKI, P. (1960). A new Arizona serotype (27: 17, 20) isolated from man. *Acta path. microbiol. scand.* **48**, 265.

HARVEY, R. W. S. (1956). Choice of a selective medium for the routine isolation of members of the salmonella group. *Mon. Bull. Minist. Hlth* **15**, 118.

HARVEY, R. W. S. (1957). The epidemiological significance of sewage bacteriology. *Br. J. clin. Pract.* **11**, 751.

HARVEY, R. W. S. & PHILLIPS, W. P. (1961). An environmental survey of bakehouses and abattoirs for salmonellae. *J. Hyg., Camb.* **59**, 93.

HARVEY, R. W. S. & PRICE, T. H. (1962). Salmonella serotypes and Arizona paracolons isolated from Indian crushed bone. *Mon. Bull. Minist. Hlth* **21**, 54.

HARVEY, R. W. S. & THOMSON, S. (1953). Optimum temperature of incubation for isolation of salmonellae. *Mon. Bull. Minst. Hlth* **12**, 149.

LOUREIRO, J. A. de (1942). A modification of Wilson and Blair's bismuth sulphite agar (stabilized stock solutions). *J. Hyg., Camb.* **42**, 224.

MOORE, B. (1948). Detection of paratyphoid carriers in towns by means of sewage examination. *Mon. Bull. Minist. Hlth* **7**, 241.

NEWELL, K. W. (1959). The investigation and control of salmonellosis. *Bull. Wld Hlth Org.* **21**, 279.

REPORT (1964). Salmonellae in abattoirs, butchers' shops and home-produced meat, and their relation to human infection. *J. Hyg., Camb.* **62**, 283.

TAYLOR, J., LAPAGE, S. P., BROOKES, M., KING, G. J. G., PAYNE, D. J. H., SANDIFORD, B. R. STEVENSON, J. S. (1965). Sources of salmonellae 1951–1963. Part I: *S. aba* to *S. muenchen*. *Mon. Bull. Minist. Hlth* **24**, 164.

J. Hyg., Camb. (1966), **64**, 275
Printed in Great Britain

Some laboratory observations on the toxicity and acceptability of norbormide to wild *Rattus norvegicus* and on feeding behaviour associated with sublethal dosing*

By J. H. GREAVES

Infestation Control Laboratory, Ministry of Agriculture, Fisheries and Food, Tolworth, Surbiton, Surrey

(*Received* 3 *December* 1965)

INTRODUCTION

Norbormide [5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidene)norborn-5-ene-2,3-dicarboximide] is reported to be selectively toxic to *Rattus norvegicus* and *R. rattus* (Roszkowski, Poos & Mohrbacher, 1964) and is therefore potentially a safe and effective alternative to existing rat poisons.

This paper describes an acute toxicity assay of norbormide and also a number of laboratory free-feeding tests using norbormide and, for comparison, zinc phosphide, primarily aimed at selecting concentrations of norbormide suitable for field testing. A secondary objective was to investigate experimentally the avoidance by rats of the two poisons at candidate field concentrations and of the bait-bases associated with them.

METHODS

All tests were carried out on individually caged wild rats of about 225 g. average body weight that had been caught by hand and kept in the laboratory for at least 4 weeks before use. Warfarin-resistant rats, that is, animals that had survived for 24 days after 6 days feeding at a normal level on 0·005 % warfarin in medium oatmeal (D. C. Drummond & E. J. Wilson, personal communication) and non-resistant rats of both sexes were used, but since there were no differences in response attributable either to sex or to resistance to warfarin, the results for these animals have been pooled.

The acute toxicity assay of norbormide was carried out on rats trained to accept dough pills made by mixing water with 90 % wholemeal flour plus 10 % caster sugar. The training involved ensuring that five pills, each of which contained approximately 0·6 g. of dry matter, were available to each rat eight times at hourly intervals each day for 4 days. Each animal was allowed a daily ration of pills amounting to a dry-matter intake of 10 % of its body weight, and when this was not eaten during the day it was left in the cage overnight. On the day of the test, after being starved overnight, each rat was given a single pill containing the poison. Forty-five female rats and forty males were tested by this method. The results for five other animals were rejected because they failed to eat the poisoned pill completely soon after it was offered.

For a rodenticide to be successful at a given concentration in bait in the field, where rats must eat a lethal dose voluntarily, it must be acceptable to them at that concentration. An attempt was therefore made to take account of acceptability as well as toxicity by carrying out three types of free-feeding test.

In the first type of test the rats were given plain food for 1 or 2 days, after which poison was added at various concentrations to the food for 1 day. The results were analysed in terms of concentration and mortality by the method of Litchfield & Wilcoxon (1949), to give estimates of lethal concentration (cf. lethal dosage).

In the second type of test, rats were prebaited with a highly palatable food consisting of 5 % caster sugar, 5 % corn oil, 65 % maize meal and 25 % rolled oats for 3 days followed by 4 days with 0·5 % norbormide in the same food. For brevity this particular food will be referred to as SCOMRO. It is believed, because of the high acceptability of SCOMRO, the period of prebaiting and the long exposure to poisoned food, that conditions were very favourable for obtaining high mortality in this test.

Free-feeding tests of the no-choice type first described are probably suitable for indicating the minimum concentration of a rodenticide suitable for use in the field but they are much less useful for comparing higher field concentrations because of the numbers of test subjects necessary to obtain significant differences at high mortality levels. However, when rats are offered a choice of plain and poisoned foods, mortality is invariably lower than in the no-choice situation. This procedure therefore offers a practicable method of comparing relatively high concentrations of poison while, at the same time, approaching nearer to conditions in the field, where unpoisoned alternative food is normally available. On this basis a series of free-feeding tests of a third type was carried out in which a choice of plain and poisoned foods was offered to each animal for an initial period of at least 2 days. Surviving animals were then given further choices, in which a variety of plain and poisoned foods were offered in pairs to find what effect exposure to poisoned food was having on subsequent choice behaviour. In all choice tests the positions of the two foods were interchanged daily to minimize the effects of place preference. During the periods between these tests, normally 3 or 5 days, the rats were given diet 41B or a new non-toxic food.

Further details of procedure are given below.

RESULTS

The bioassay

The results of the acute toxicity assay (Table 1) were analysed by the method of Litchfield & Wilcoxon (1949) to give an LD50 for norbormide of 9·0 mg./kg. (95 % limits, 10·9–7·4 mg./kg.) and an LD95 of 17·0 mg./kg. This estimate of the LD50 may be compared with the figure of 12 mg./kg. reported by Roszkowski *et al.* (1964) for wild *R. norvegicus* dosed with an acid solution of the compound by stomach tube.

Among twenty-two animals whose behaviour was recorded, signs of poisoning, including locomotor impairment, tail lashing, laboured breathing and occasional,

usually terminal, convulsions were observed from 15 min. after dosing. All deaths occurred within 24 hr., the earliest being in 40 min.

No-choice tests

The results of the free-feeding tests of the first type are summarized in Table 2. The estimates of dosage must be treated with reserve since, for individual rats, the weighings involved in estimating the amounts of bait eaten overnight were subject to errors that may have produced inaccuracies of the order of 20–30 mg./kg. for a poison concentration of 2·5 % and proportionately less for lower concentrations.

Table 1. *Mortality in rats dosed with norbormide by pill*

Dosage (mg./kg.)...	2	4	8	10	11	12	13	16	20	22	24
Mortality	0/6	0/9	7/19	3/4	5/5	7/10	4/5	18/19	3/3	1/1	4/4

Table 2. *Mortality in rats fed norbormide or zinc phosphide in medium oatmeal for* 1 *day after* 1–2 *days of prebaiting*

Poison	Concentration (%)	Mortality	Mean and range of dosages that killed (mg./kg.)	Highest dosage survived (mg./kg.)
Norbormide	0·01	0/13	— —	14
	0·05	2/10	11 (4–18)	33
	0·07	1/13	28 —	58
	0·1	1/12	19 —	26
	0·25	5/13	24 (1–59)	154
	0·5	18/28	25 (0–68)	69
	0·8	6/9	87 (0–294)	41
	1·0	15/17	89 (13–763)	22
	2·0	11/11	56 (16–136)	—
Zinc phosphide	0·1	0/4	— —	45
	0·2	2/4	36 (27–45)	59
	0·4	1/4	28 —	70
	0·5	3/4	57 (35–86)	24
	0·6	2/4	63 (58–68)	99
	1·0	5/5	57 (0–111)	—
	2·5	4/4	40 (0–109)	—

The figures illustrate, however, that there is no close relationship between dosage and either concentration or mortality. In contrast, there is quite a strong association between concentration and mortality. Analysis of the results in Table 2 gives a median lethal concentration (LC50) for zinc phosphide in medium oatmeal of 0·44 % (95 % limits, 0·27–0·81 %) and for norbormide in the same bait 0·37 % (95 % limits, 0·24–0·57 %). The respective LC95's, which are perhaps of greater practical interest, are 1·3 and 3·4 %.

The second type of test involving 0·5 % norbormide in SCOMRO, in which, as stated earlier, conditions favourable for a good kill were provided, did not result (Table 3) in a marked increase in mortality over that obtained at the same concentration in less extreme conditions (Table 2). The four animals in Table 3 that survived ate negligible amounts during their 4-day exposure to the poisoned food.

Choice tests

Responses to an initial choice between similar foods

In the third type of free-feeding test each rat was offered plain and poisoned, but otherwise identical foods. After 2 nights' feeding surviving animals were assigned to one of two categories (Table 4). These were 'refusers' which had eaten very little food and 'discriminators' which had eaten relatively large amounts of unpoisoned food. The distinction between refusers and discriminators was obvious except in a few borderline cases. The mean daily consumption of unpoisoned food by the sixteen refusers in Table 4 was 11·0 g./kg. compared with a mean of 59·3 g./kg. eaten by the discriminators. Both types of survivor ate significantly less food

Table 3. *Cumulative mortality in rats fed* 0·5 % *norbormide in SCOMRO* for* 4 *days after* 3 *days of prebaiting*

Day	1	2	3	4
Mortality	17/24	18/24	18/24	20/24

* See text.

Table 4. *Rats classified by response made when offered a choice of poisoned food and the same food unpoisoned*

Group	Type of rat *vis à vis* the poison	Food	Poison	Poison in food (%)	Total no. of rats	Dead (days 1 and 2)	Refusers (surviving 2 days)	Dis-criminators (dying on days 3 and 4)	Dis-criminators (surviving 4 days)
1	Naïve	Medium oatmeal	Norbormide	0·5	12	3	6	2	1
2	Naïve	Medium oatmeal	Norbormide	1·0	12	4	2	2	4
3	Naïve	Medium oatmeal	Norbormide	2·0	12	4	1	1	6
4	Naïve	Medium oatmeal	Zinc phosphide	1·0	12	2	0	0	10
5	Naïve	Medium oatmeal	Zinc phosphide	2·5	12	6	1	0	5
6	Naïve	Medium oatmeal	Zinc phosphide	5·0	12	7	0	0	5
7	Experienced	Medium oatmeal	Norbormide	1·0	12	2	2	1	7
8	Experienced	Sausage rusk	Norbormide	1·0	16	4	4	2	6
9	Experienced	Medium oatmeal	Zinc phosphide	1·0	11	0	0	0	11

($P < 0{\cdot}001$) than a control group without access to poisoned food, in which each animal ate an average of 105·9 g./kg. daily. This depression of feeding among the experimental animals was presumably one of the effects of the poisoned food of which they ate on average 3·2 g./kg. daily. The test was ended for refusers after 2 days so as to avoid deaths that could have been due to starvation, but was continued for a further 2 days with discriminators. There were therefore two categories of mortality: animals dying in the first 2 days of the test, and those that died in the second 2 days after discriminating for the first two (Table 4).

Some animals had previously experienced the same poison in either the bioassay or the no-choice tests and the results for these are separated in Table 4 from those for the rats that had had no experience of the poison. The only obvious difference

between the results for naïve and experienced rats offered norbormide or zinc phosphide at the same concentration is that mortality was lower among the experienced animals, which would be expected on the grounds that resistance to the poisons had been selected for in these groups. However, only the results obtained with the experimentally naïve animals are considered further.

Taking first the results for norbormide in medium oatmeal, the mortality is very similar at each of the concentrations tested, suggesting that in the field they may not differ much in effectiveness. The association ($P < 0{\cdot}025$) between the ratio of surviving discriminators to refusers and concentrations of poison in the food (1/6, 4/2, and 6/1 at 0·5, 1·0 and 2·0 % respectively) suggests that the taste of norbormide caused aversion or was more easily associated with the toxic effects of the poison at increasing concentrations. A similar apparent association between the proportion of discriminators surviving the third and fourth days and the concentration of norbormide in the food (1/3, 4/6 and 6/7 at 0·5, 1·0 and 2·0 % respectively), though not statistically significant, does not conflict with the idea that discrimination of plain from poisoned food was less easy at lower than at higher concentrations. The refusers may have been animals that associated the toxic effects of the poison with the taste of the food rather than the taste of the poison. This interpretation is supported (Table 8) by the comparative rarity of refusers in choice tests where the food containing the poison was different from the unpoisoned food, thus providing additional cues calculated to make discrimination easier. Another possibility is, however, that some of the refusers were too sick to eat because of sublethal feeding on the poisoned food. This is supported by the fact that the average daily consumption (94·4 g./kg.) of a new food on days 3 and 4 by fifteen refusers was lower than that of the control group that was not exposed to poisoned food.

With zinc phosphide, the increasing mortality with concentration suggests that 5 % may be the best of the three concentrations for field use. It also indicates that with zinc phosphide, unlike norbormide, the tendency for increased concentration (through the ranges tested) to produce higher mortality is not offset by decreased palatability or increased speed of action, both of which might be expected to reduce the chances of a lethal dose being ingested. It is apparent, however, from the high proportion of discriminators among the survivors of the first 2 days of the test and from the fact that none died in the second 2 days, that zinc phosphide has a distinct taste to rats.

Second and third choice tests on survivors

All the surviving discriminators of Table 4, except for seven (from Group 9) that had been given a choice involving 1 % zinc phosphide, were given second and third choice tests, in which the poison employed was always of the same kind and at the same concentration as in the first choice. For their second test the discriminators were offered a choice for 4 days of the food given in the first test and a new food, one of which was poisoned. It was found that the rats either died in the first 24 hr. of the test or they ate, as before, mainly the unpoisoned food throughout. The chief interest in the results (Table 5) lies in the significantly higher mortality, irrespective of the poison, where this was presented in medium oatmeal ($P < 0{\cdot}001$).

Since a control group strongly preferred medium oatmeal to sausage rusk ($P < 0{\cdot}001$) it appears that many animals died through eating poisoned medium oatmeal because the alternative was less palatable. However, the data in Table 5 do not suggest that the previous experience of some of the rats in discriminating against the same poison at the same concentration in the same food, had any marked effect on their choice of behaviour in this test.

Table 5. *Mortality in discriminators in the second test in relation to the foods offered in the first and second tests*

			Second test		
Group	Poison	Food in first test	Plain food	Poisoned food	Mortality
10	Norbormide	MO*	SR	MO	8/9
11	Norbormide	SR†	SR	MO	2/3
12	Zinc phosphide	MO	SR	MO	8/13
13	Norbormide	MO	MO	SR	0/9
14	Norbormide	SR	MO	SR	1/3
15	Zinc phosphide	MO	MO	SR	1/11

* Medium oatmeal. † Sausage rusk.

Table 6. *Preferences of discriminators, offered a choice of medium oatmeal and sausage rusk, in relation to the foods encountered in the second test*

		Percentage preference of individual rats for medium oatmeal					
Group	Foods offered in the second test						
16	SR* *v.* MO† + norbormide	0·3	0·2	—	—	—	—
17	SR *v.* MO + zinc phosphide	97·9	34·8	2·7	0·9	0·4	—
18	MO *v.* SR + norbormide	100·0	100·0	100·0	100·0	100·0	92·6
		80·4	54·6	2·3	—	—	—
19	MO *v.* SR + zinc phosphide	100·0	100·0	100·0	100·0	100·0	100·0
		99·2	99·1	72·8	71·2	—	—
20	Controls (experimentally naïve)	99·9	99·7	99·5	98·9	98·5	93·1
		92·9	90·5	79·6	67·2	57·6	—

* Sausage rusk. † Medium oatmeal.

In their third test twenty-six out of the twenty-eight survivors in Table 5 were given a choice for 4 days of medium oatmeal and sausage rusk (both unpoisoned) to check whether they would show a preference, as compared with a control group, for the food that was unpoisoned in the second test. The results (Table 6) show that there was little, if any, difference between the two poisons in their effect on food preferences in this test. All but one of the rats that had experienced sausage rusk as non-toxic in the second test (Groups 16 and 17) showed, in contrast to the controls, a clear preference for this food. Any corresponding trend among the rats that had experienced medium oatmeal as non-toxic (Groups 18 and 19) is less obvious owing to the strong preference for medium oatmeal among the control animals. However, the high proportion of animals in Groups 18 and 19 that ate medium oatmeal exclusively, suggests that such a trend was present.

The aberrant animal in Group 18 that ate only 2·3 % of its food as medium oatmeal had shown anomalous behaviour before. After discriminating against 1 % norbormide in sausage rusk in the first test it had gone on to eat 19·3 g. of similarly poisoned sausage rusk in the second test as against only 4·3 g. of plain medium oatmeal.

Turning now to the refusers in Table 4, all sixteen were given, in their second test, a 4-day choice of medium oatmeal and sausage rusk, both unpoisoned, to check whether in comparison with the same control group they had learned to avoid the food offered in the first test. The results are given in Table 7 and show that, as with the discriminators, there were clear shifts in preference towards the food not experienced as toxic only when this was sausage rusk. The almost complete avoidance of medium oatmeal shown by six rats in Groups 21 and 22 compared with the behaviour of the controls indicates that the refusal of these animals to feed in the first test was a learned response in which they were using the taste of medium oatmeal as a cue.

Table 7. *Preferences of refusers, offered a choice of medium oatmeal and sausage rusk, in relation to the foods encountered in the first test*

Group	Foods offered in the first test	Percentage preference of individual rats for medium oatmeal					
21	MO* *v.* MO + norbormide	99·1	96·9	88·1	71·4	70·2	47·5
		1·5	0·6	0·3	0·3	0·0	—
22	MO *v.* MO + zinc phosphide	2·4	—	—	—	—	—
23	SR† *v.* SR + norbormide	100·0	100·0	100·0	59·3	—	—
24	Controls (experimentally naïve)	99·9	99·7	99·5	98·9	98·5	93·1
		92·9	90·5	79·6	67·2	57·6	—

* Medium oatmeal. † Sausage rusk.

At this point the rat in Group 5 of Table 4 that originally refused both medium oatmeal and 2½ % zinc phosphide in medium oatmeal was given this choice again and repeated its refusal. The remaining fifteen animals, all of which had been exposed to norbormide, were given the same test as in Table 4 but with 85 % coarse oatmeal, 10 % caster sugar and 5 % mineral oil, as the bait-base. Only two rats repeated their refusal; three others died in the first 2 days and the other ten discriminated against the poisoned food. This change to discriminatory feeding may have resulted in part from the extra practice the rats had had in choice situations. However there is evidence (see later) that some animals may have formed a discrimination in the original test that went undetected because, soon after its formation, food consumption was reduced as a result of illness.

Tests in which the initial choice lay between different foods

The results of the second test with discriminators (Table 5) had indicated that, in the choice situation, the more palatable the poisoned food as compared with the unpoisoned alternative, the higher the mortality was likely to be. The effect of palatability was therefore further investigated in two groups of experimentally

naïve animals. One group (25) was offered a choice between 0·5 % norbormide in SCOMRO and plain sausage rusk for a minimum of 4 days while the other (group 26) was given a 4-day choice between 0·5 % norbormide in sausage rusk and unpoisoned SCOMRO. Table 8 shows that mortality was significantly higher ($P < 0{\cdot}01$) where the poison was in the more palatable SCOMRO.

Among the discriminators in Group 25 of Table 8 one rat showed unusual tolerance of norbormide. The test was therefore continued with this animal for 11 consecutive days, during which it ate a total of 68·6 g. of poisoned food and 98·4 g of plain food and survived.

Meanwhile, five of the other sixteen survivors of Group 25 (two discriminators and three refusers) were given the same choice again. This time all five survived by discriminatory feeding. At the same time the remaining eleven animals of Group 25 (eight discriminators and three refusers) were offered a 4-day choice of SCOMRO and sausage rusk, both unpoisoned. The preferences of the animals in this test, and those of an experimentally naïve control group are given in Table 9.

Table 8. *Rats classified by response when offered a choice of two foods, one of which contained 0·5 % norbormide*

Group	Poisoned food	Plain food	Total number of rats	Refusers (surviving 2 days)	Discriminators (surviving 4 days)	Dead
25	SCOMRO*	SR	44	6	11	27
26	SR†	SCOMRO	16	0	14	2

* See text. † Sausage rusk.

Table 9. *Preferences of rats offered a choice of sausage rusk and SCOMRO* after exposure to a choice of sausage rusk and norbormide-poisoned SCOMRO*

Group	Percentage preference of individual rats for sausage rusk							
Experimental	98·6	98·6	98·3	98·2	98·2	93·2	89·2	59·0
	49·8	13·5	5·0	—	—	—	—	—
Control	30·7	10·1	5·5	4·7	4·7	4·6	3·2	2·2
	0·0	0·0	—	—	—	—	—	—

* See text.

There can be little reason to doubt that the marked difference between the preferences of the experimental and control animals resulted from the prior exposure of the experimental rats to norbormide-poisoned SCOMRO.

Since the three refusers all ate virtually only sausage rusk, it seems most likely that all six of the refusers in Table 8, by the time they were classified as such, had been conditioned to avoid eating SCOMRO in favour of the normally less palatable sausage rusk.

DISCUSSION

Two methods of calculating the optimum field concentration of a rodenticide from laboratory data have been discussed by Bentley (1958). The first of these is based on evidence collected by Thompson (in Chitty & Southern, 1954) that, in the field, rats eat on average at least 1 % of their body weight in the form of dry poisoned cereal bait, and that for successful control treatments it is necessary that this amount of bait should contain about eight LD 50's. Using the figure of 9 mg./kg. as the LD 50 of norbormide and 41·3 mg./kg. for zinc phosphide (Chitty & Southern, 1954), this method indicates field concentrations of 0·72 % and 3·3 % for the two poisons respectively. In the second method more weight is given to the slope of the dosage-mortality curve by allowing one LD 95 in the same quantity of bait and multiplying the percentage thus arrived at by three to allow for particularly resistant animals and for rats eating less than average amounts of bait. Adopting an LD 95 for norbormide of 17·0 mg./kg. and for zinc phosphide 73·0 mg./kg. (calculated from data given by Chitty & Southern, 1954) this procedure yields estimates of 0·51 and 2·2 % respectively.

While empirically based, both these methods rest on the assumption that differences among rodenticides in their palatability and speed of action result in negligible differences in consumption of poison bait by rats in the field. Though this may possibly be so for the poisons involved in Thompson's observations (zinc phosphide, antu, arsenic, red squill and barium carbonate) it is not necessarily true of other rodenticides. For this reason the results of the free-feeding tests are of interest. Here, the slope of the probit mortality/log concentration line calculated from the results of the no-choice tests for zinc phosphide is significantly steeper than that for norbormide (4·14 as compared with 1·63: $P < 0·05$) and therefore, in comparison with norbormide, the likelihood of a lethal dose of zinc phosphide being eaten rises at a faster rate with increasing concentration. Further, this difference almost certainly resulted from reduced acceptance offsetting increases in the toxicity of the baits to a greater extent with norbormide than with zinc phosphide. This follows from the fact that the slopes of the probit mortality/log dosage lines based on bioassay data for norbormide and zinc phosphide (6·25 and 6·61 respectively) are steeper, yet more nearly equal. The same effect has already been commented upon in relation to the results of the choice tests—which indicate that the effectiveness of norbormide is likely to be about equal at all concentrations from 0·5 % to 2·0 % while that of zinc phosphide may be expected to increase up to a concentration greater than 2·5 %.

The marginally lower mortality obtained in the choice tests with norbormide as compared with 5·0 % zinc phosphide suggests that the latter might give the better results if both were used in the same conditions in the field. However, since the rats discriminated less consistently against norbormide than against zinc phosphide and because, in the field, the specificity of norbormide removes limitations on the mode of distribution of this poison which must still apply to zinc phosphide, it seems possible that norbormide at 0·5 % or more may give results as good as or better than 5·0 % zinc phosphide, particularly when used over periods

of several days without prebaiting. Nevertheless, the failure of 0·5 % norbormide to give a complete kill (Table 3) in apparently favourable laboratory conditions indicates that, like zinc phosphide, it would not often give complete kills in single treatments against sizeable infestations in the field. Further, if the resistance shown by a few individuals is heritable, and if norbormide was used extensively, then a general reduction in its effectiveness would result as resistance to the poison became prevalent.

The amounts eaten in the original choice tests showed that the discriminators avoided eating a lethal dose by reacting to the taste of the poison. It was not possible however to determine whether the taste of poison caused aversion initially, or whether it did so later through being associated with symptoms of poisoning. In either event, the increased mortality in the tests in which poison was presented in the more palatable of two foods showed that aversion to the poisons could be overcome to a marked extent by using a highly palatable bait-base. The refusers, animals that apparently did not use the taste of poison as a means to identify the unpoisoned bait, survived by virtually not feeding at all. In some cases almost certainly, this was simply owing to illness. In others however it seemed to be primarily behavioural, since several animals showed marked shifts of preference away from the food experienced as toxic and towards a normally less acceptable food (Table 7). Similarly, Tables 6 and 9 show that some animals, after surviving exposure to a choice of a palatable food containing poison, and an unpoisoned though less palatable alternative, later showed a conditional shift of preference towards the less palatable food. This result is consistent with the concept of 'bait shyness' defined by Rzoska (1953) as 'a cautious attitude towards food (and poison bait) experienced previously with harmful effects'. Thus it seems likely that in the field, when attempting to eliminate survivors of norbormide treatments, it will usually be necessary, as with zinc phosphide, to use a different poison and bait-base.

The inferences drawn here as to the practical significance of the results must, however, remain tentative until they have been checked against experience in the field. In particular, it would be useful to carry out comparative field trials aimed at assessing the validity of laboratory methods of evaluating quick-acting rodenticides. This applies especially to the choice test, which at present seems to be more capable of further development than other methods, and more sensitive to the varied factors affecting a compound's rodenticidal efficiency.

SUMMARY

The median lethal dose of orally administered norbormide for wild *Rattus norvegicus* was found to be 9·0 mg./kg. of body weight and the LD 95 about 17·0 mg./kg.

In tests in which various concentrations of norbormide or zinc phosphide were added to the food of individually caged wild rats, mortality increased with concentration of poison, though more slowly with norbormide than with zinc phosphide. The mortality that occurred among rats offered a choice between unpoisoned food and the same food with added norbormide or zinc phosphide indicates that in control treatments in the field the optimum concentration of norbormide in

bait would be about 0·5 % and that this might be expected to give results comparable with those obtainable with 2·5 % or 5·0 % zinc phosphide. Other methods of estimating suitable field strengths indicate that concentrations of norbormide higher than 0·5 % may be preferable.

Some animals that survived exposure to a choice of plain food and the same food poisoned with norbormide or zinc phosphide at field concentrations avoided eating lethal amounts by reacting to the taste of the poison. Others learned to use the taste of the food, not that of the poison as a cue and later avoided eating the food when it contained no poison.

When either poison was presented to rats in the more palatable of two foods in the choice situation mortality was relatively high. Some of the surviving animals subsequently rejected the more palatable food in preference to the normally less palatable alternative.

I am indebted to Tavolek Laboratories Ltd., Slough, who supplied the norbormide used in these experiments free of charge.

REFERENCES

Bentley, E. W. (1958). Biological methods for the evaluation of rodenticides. *Tech. Bull. Minist. Agric. Fish. Fd*, no. 8.

Chitty, D. & Southern, H. N. (1954). *Control of Rats and Mice.* Oxford: Clarendon Press. 3 vols.

Litchfield, J. T. & Wilcoxon, F. (1949). A simplified method of evaluating dose-effect experiments. *J. Pharmac. exp. Ther.* **96**, 99–113.

Roszkowski, A. P., Poos, G. I. & Mohrbacher, R. J. (1964). Selective rat toxicant. *Science, N.Y.*, **144**, 412–3.

Rzoska, J. (1953). Bait shyness, a study in rat behaviour. *Br. J. Anim. Behav.* **1**, 128–35.

J. Hyg., Camb. (1966), **64**, 287
Printed in Great Britain

Some studies on the epidemiology of Sonne dysentery. Changes in colicine type and antibiotic resistance between 1956 and 1965

By W. N. FARRANT and A. J. H. TOMLINSON
Public Health Laboratory, County Hall, London, S.E. 1

(*Received* 29 *December* 1965)

INTRODUCTION

The epidemiology of Sonne dysentery presents a number of problems. In large urban areas the infection is endemic and it spreads, in larger or smaller epidemic episodes, to smaller areas of population. The infecting dose is small and infection is principally transmitted by direct or indirect contact (Hutchinson, 1956); food-borne outbreaks are uncommon. There is a well-marked seasonal variation in frequency with a peak in the early spring (Bradley, Richmond, Shaw & Taylor, 1958) and there is no obvious immunity following infection. It was hoped that the use of colicine typing (Abbott & Shannon, 1958; Abbott & Graham, 1961) and the further characterization of strains of *Shigella sonnei* that was possible by determining their antibiotic sensitivities might throw light on the spread of Sonne dysentery by enabling one to follow accurately the spread of infection from individual to individual.

Recent work has shown that the factors responsible for antibiotic resistance and for colicine production may be transferred from organisms possessing these properties to others that lack them. This transfer is not confined to members of a single species but can occur between organisms of many genera within the family Enterobacteriaceae (Datta, 1962; Watanabe, 1963; Ozeki, Stocker & Smith, 1962).

Anderson & Lewis (1965) have shown that an organism might carry a transfer factor, a resistance determinant, both, or neither. Organisms possessing both the transfer factor and a resistance determinant were able to convert a proportion of suitable sensitive organisms to resistance. Organisms carrying the resistance determinant alone were resistant to the antibiotic but unable to transfer this resistance unless it was mobilized by a transfer factor from some other source. Sensitive organisms carrying the transfer factor alone infected a high proportion (10–50 %) of other organisms with this factor without changing their resistance. Ozeki *et al.* (1962) showed that some factors responsible for colicine production could be transferred to strains that lacked them, and Anderson & Lewis (1965) found that the transfer factors responsible for the transfer of antibiotic resistance and for colicine transfer were mutually replaceable.

Usually resistance or colicine factors were transferred only to a small proportion (*ca.* 1/1000) of susceptible cells but Anderson & Lewis (1965) describe two resistance determinants which were not separable from the transfer factor and which infected—and gave resistance to—a high proportion (*ca.* 50 %) of susceptible cells.

The factors *Col 1* and *Col B* of Ozeki *et al.* (1962) could also be transferred to a high proportion of susceptible cells and such transfers clearly could change the character of an intestinal organism in the absence of special conditions (e.g. antibiotic therapy) favouring the organism with newly acquired characters.

A few experiments in our laboratory have shown that both antibiotic resistance (to sulphonamide, streptomycin and tetracycline) and colicine production can be transferred to strains of *Sh. sonnei* from strains of *Escherichia coli* derived from normal faeces possessing these properties and from *Sh. sonnei* to susceptible strains of *E. coli*. The exact details have not yet been worked out but it seems likely that they will conform to the model described by Anderson & Lewis (1965).

METHODS AND MATERIALS

Source of materials

Specimens of faeces were received for bacteriological diagnosis from General Practitioners and Medical Officers of Health in the London area. The home address of the patient made it possible to assign each isolation to a Local Authority area but the source of infection might have been elsewhere. Approximately 90 % of the isolations of *Sh. sonnei* were derived from fifteen boroughs, namely, Islington, Hackney, Stoke Newington and Shoreditch in North and North-East London, Chelsea, Kensington and Hammersmith in West London, Wandsworth, Battersea, Lambeth, Camberwell, Southwark and Bermondsey which made up the whole of the London County Council area south of the River Thames and two county boroughs outside the central area, West Ham and Croydon. The remainder of the isolations were derived from a number of other boroughs in Central London but specimens were not received regularly and in large numbers from them over the whole period of observation. Some of the analyses of the results have been confined to the fifteen 'major' boroughs. No specimens were examined from Bermondsey during the first 18 months of observation nor from Croydon during the last 12 months. The results obtained from large residential institutions in which Sonne dysentery was often endemic for long periods have not been included.

In October 1956 Dr J. D. Abbott made available to us his method of colicine typing and sent us his indicator strains. This enabled us to start routine colicine typing before the method was published and we are extremely grateful to him for this courtesy.

On 1 April 1965, the administrative structure of London was reorganized, with the merging of boroughs and changes in boundaries. This seemed an appropriate moment to conclude this particular series of observations.

Isolation and identification of Shigella sonnei

Specimens of faeces were plated on deoxycholate citrate agar and inoculated into selenite broth which was plated on the same medium after 24 hr. incubation. Identification of *Sh. sonnei* was based on preliminary slide agglutination followed by biochemical and serological confirmation.

Selection of strains of Shigella sonnei *for further investigation*

It was not possible to investigate the resistance pattern and colicine type of every strain isolated since the numbers were too great. No strains from convalescent patients, i.e. those known to have been previously infected, have been examined. So far as fresh infections were concerned, our policy has been to investigate one strain from each incident—an incident being defined as a number of infections that might be expected to have a single source. In practice this has meant that one strain has been investigated from infections in families, in day nurseries and in small residential institutions. In fact, we have often examined several strains from larger outbreaks in day nurseries but, in order not to load the results, only the first strain has been included in this analysis. It has not always been possible to examine every strain from sharp outbreaks in primary schools but otherwise we have been able to maintain our policy of examining one strain from each incident.

Tests for sensitivity to antibiotics

A single colony was picked from the primary deoxycholate citrate plate into peptone water containing 1 % of lactose and sucrose and an indicator, and on to an agar slope. After the peptone water cultures had been incubated for 4–6 hrs. at 37° C., sensitivity tests were put up by streaking small loopfuls of culture, usually 8 to a plate, to a single disk in the centre containing the appropriate antibiotic. Tests for sulphonamide sensitivity were carried out on lysed blood agar plates to a disk containing 300 μg. sulphathiazole; tests for sensitivity to streptomycin and terramycin on nutrient agar plates to disks containing 25 and 50 μg. respectively. Tests were read after overnight incubation and checked for purity. Neomycin has been used throughout this survey but only occasional strains have been found resistant to it. Ampicillin and chloramphenicol have been used from time to time; about 50 % of strains were resistant to the former, occasional strains to the latter.

Colicine typing

The method did not differ materially from that described by Abbott & Shannon (1958), in which the strains of *Sh. sonnei* were grown on tryptose soya agar (Oxoid) containing 5 % horse blood and nine indicator strains were used to detect colicine production.

We have found that the colicines produced by some strains of *Sh. sonnei* are somewhat thermolabile and may be partly destroyed by incubation, as recommended by Abbott & Shannon, for 3 days at 37° C. Maximum colicine production appears to occur after overnight incubation at 33–35° C. and this temperature is to be preferred.

Our experience agrees with that of Abbott & Graham (1961) that colicine type 11 should not be separated from type 6. When incubated at 33–35° C., all strains of type 11 have given the inhibition pattern of type 6. During the course of this work we have isolated five strains of *Sh. sonnei* whose inhibition patterns did not allow them to be assigned to any colicine type. Since each occurred once only, they have been excluded from this analysis.

RESULTS

During the 8½ years under review (October 1956 to March 1965) *Sh. sonnei* was isolated from 19,859 infected persons. The results of colicine typing and antibiotic resistance of 9419 of these strains are analysed here on the basis of one strain from each incident as previously defined. In a large urban area, such as London, the total incidence of Sonne dysentery is made up of the sum of several local prevalencies. We have often observed the rise and fall of local epidemics; sometimes these have been isolated events in the sense that there was no increased frequency of infection

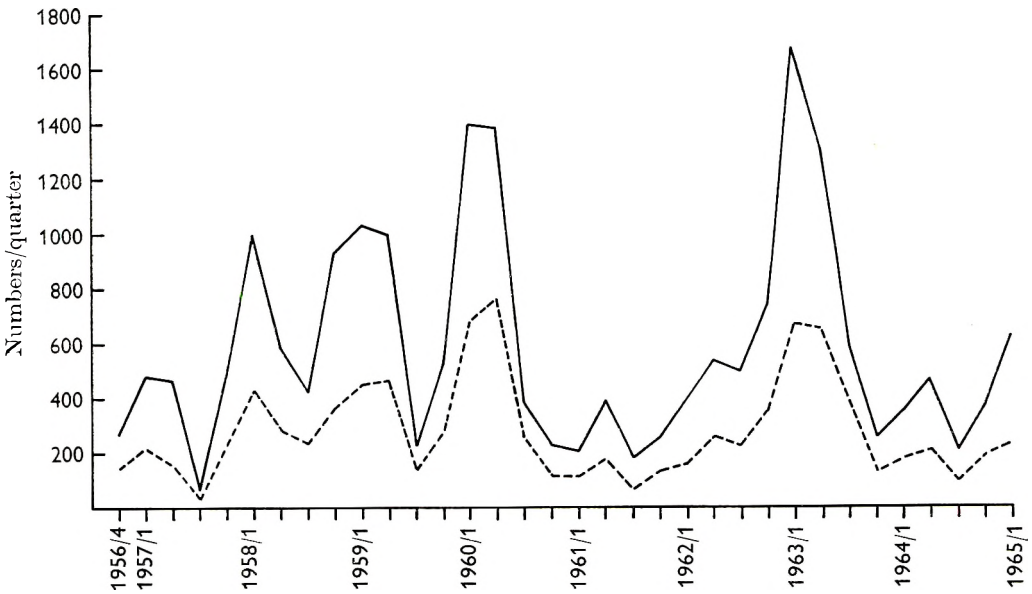


Fig. 1. The quarterly number of incidents and new isolations of *Sh. sonnei*, 1956–65. ——, New isolations; - - -, incidents.

elsewhere, at other times local epidemics of varying severity have occurred more or less simultaneously in several areas. In each of the fifteen boroughs from which large numbers of specimens have been regularly examined, the highest number of new infections in any one quarter has been at least 30 times, and in several boroughs 100 times, greater than the lowest quarterly total of new infections.

The number of incidents and the total number of new isolations of *Sh. sonnei* from October 1956 to March 1965 for each period of 3 months (quarter) are shown in Fig. 1.

This figure shows a series of winter peaks and summer troughs—the usual distribution of Sonne dysentery in Great Britain at the present time. In some winters there was little real increase in the incidence of dysentery. It will also be seen that the ratio of incidents to new isolations remained roughly constant throughout the observation period.

The distribution of colicine types is shown in Fig. 2 by 3-monthly periods over the 8½ years that observations have been made. Strains of *Sh. sonnei* that produce

no detectable colicines are described as type 0 by some workers and 'untypable' by others. Attempts, since the original description of the technique by Abbott & Shannon (1958), to demonstrate colicine production by these strains have all proved fruitless and it has not proved possible to divide up this group in other ways. It seems that these strains are best regarded as producing no colicines and

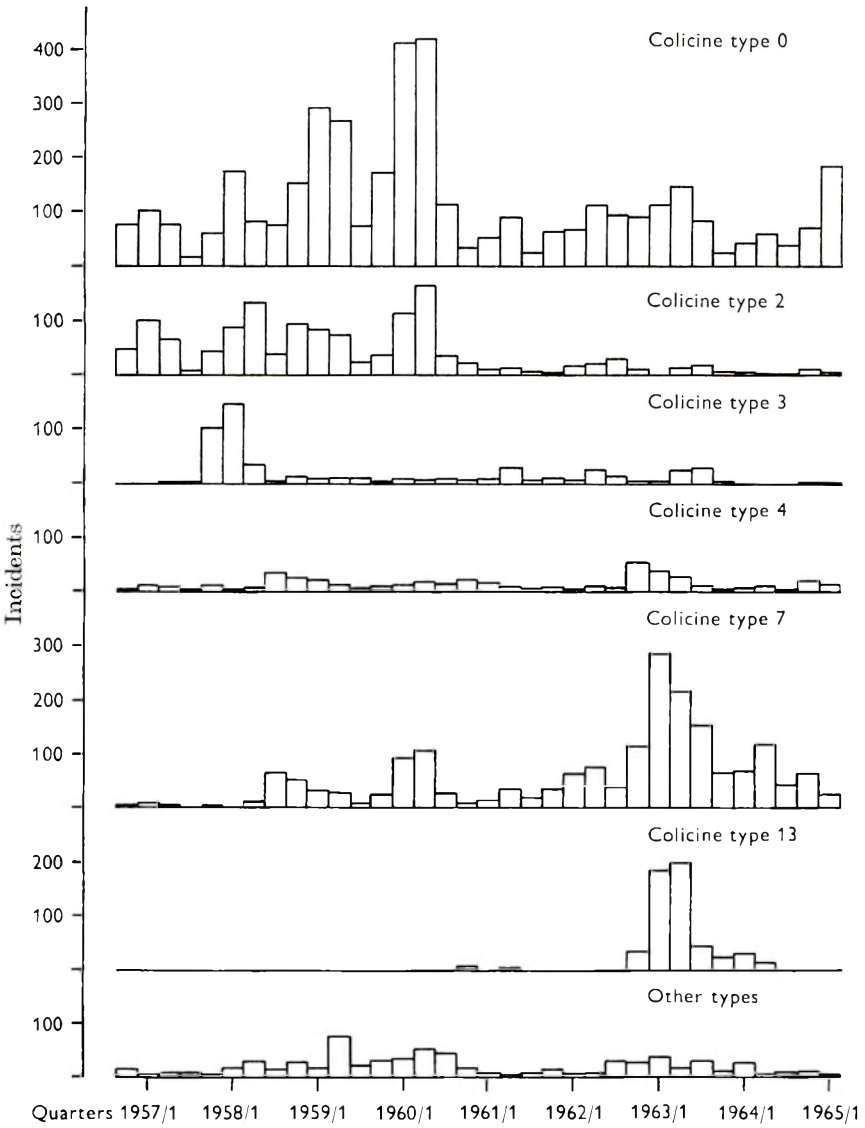


Fig. 2. The quarterly distribution of colicine types of *Sh. sonnei*, 1956–65.

that type 0 is therefore the better name. Nearly half our strains, 3997 out of 9419, produced no colicines; five colicine types, types 2, 3, 4, 7 and 13, were responsible for 4760 incidents. Other colicine types caused 662 incidents, 7 % of the total.

It will be seen from Fig. 2 that three of the six commonly observed colicine types, types 0, 2 and 4, were present in the area and causing infections throughout the

observation period, but that the other three common types were absent, or virtually so, at the start of the period, gave rise to outbreaks of epidemic proportions and then declined. This sequence is seen most clearly with colicine type 13. No strains of this type were found during the first 4 years, six strains were isolated in West Ham during the 4th quarter of 1960 and a single strain from central London during 1961. Late in 1962, 18 months after the last previous isolation, an epidemic due to this type was observed and by the middle of 1964 a total of 560 incidents due to this type had been recorded; no strains of this type were isolated after June 1964.

The first strains of colicine type 13 that were isolated in 1962 all had the same pattern of antibiotic resistance. They were all resistant to sulphonamides but sensitive to streptomycin and the tetracyclines. A single strain sensitive to sulphonamides was isolated in the last week of 1962 and early in 1963 strains with different patterns of antibiotic resistance were isolated from scattered incidents. Details are given in Table 1.

Table 1. *Number of incidents due to* Shigella sonnei *colicine type* 13

		Antibiotic resistance pattern				
Sulphonamide		S	R	R	R	R
Streptomycin		S	S	R	S	R
Tetracycline		S	S	S	R	R
Year	Quarter					
1962	4	1	36	0	0	0
1963	1	1	175	2	5	0
	2	4	146	1	10	38
	3	4	45	2	13	2
	4	0	4	0	12	9
1964	1	0	1	1	9	23
	2	0	0	0	2	14
	3	0	0	0	0	0

It will be seen that the original epidemic strain had virtually died out, except for five scattered incidents, within 12 months of its original introduction and the epidemic was continued by tetracycline resistant strains that might or might not be sensitive to streptomycin. These in their turn disappeared. The epidemic was largely confined to a group of four contiguous boroughs in North East London. Only fifteen of the 560 incidents occurred outside this area and these did not spread locally.

Altogether strains of this type of *Sh. sonnei* showing five different resistance patterns were encountered. During the first 6 years observation this colicine type was introduced into the area on two occasions; it seems unlikely that introductions of this type with five different resistance patterns should occur over a period of 6 months and that by chance these introductions should all be into that area of North East London where type 13 was already established. It is much more likely that the antibiotic resistance pattern of this organism changed after a single introduction late in 1962.

A rather similar sequence of events can be traced with colicine type 7 though the process has proceeded more slowly and the final elimination of the type had not occurred by March 1965. Details of the antibiotic resistance patterns of all the strains of this type are given in Table 2.

Table 2. *Number of incidents due to* Shigella sonnei *colicine type* 7

		Antibiotic resistance pattern							
Sulphonamide		S	S	S	S	R	R	R	R
Streptomycin		R	R	S	S	S	R	S	R
Tetracycline		R	S	R	S	S	S	R	R
Year	Quarter								
1956 to 1958	4 to 1	.	.	.	1	4	.	.	.
	2	.	.	.	.	10	.	.	.
	3	.	.	.	.	64	.	.	.
	4	.	.	.	.	50	1	.	.
1959	1	.	.	.	2	25	1	3	.
	2	.	.	.	5	22	1	2	.
	3	.	.	.	.	5	1	1	.
	4	.	.	.	5	8	12	.	.
1960	1	.	.	2	42	39	9	.	.
	2	1	2	1	62	33	3	2	.
	3	.	.	.	19	6	1	.	.
	4	.	.	.	5	2	.	.	.
1961	1	.	.	.	8	4	4	.	.
	2	.	.	.	6	30	.	1	.
	3	.	.	.	3	10	1	5	.
	4	.	.	.	.	34	.	.	.
1962	1	.	.	.	1	59	1	4	.
	2	.	.	.	3	69	.	3	2
	3	.	.	.	3	35	.	1	.
	4	.	.	1	25	82	1	4	1
1963	1	.	.	.	23	235	8	14	6
	2	.	1	1	11	161	28	12	4
	3	.	.	1	6	129	9	4	3
	4	.	.	1	4	29	13	12	3
1964	1	.	.	.	3	25	6	26	8
	2	.	.	.	3	26	54	31	4
	3	.	.	.	.	8	20	14	3
	4	.	.	.	.	10	4	47	4
1965	1	.	.	.	.	6	1	17	2

The first five strains of this type were isolated over a period of 18 months from five separate boroughs. In the second quarter of 1958 a sulphonamide resistant strain appeared in Islington—the first type 7 in this borough—and spread at first locally and later over the whole area. At the beginning of the epidemic in 1958 the strains of type 7 all had the same pattern of resistance but later a wide variety of resistance patterns was observed. Strains with the original resistance pattern nearly disappeared in 1959 and in 1960 and were uncommon towards the end of

the period of observations. Table 2 can perhaps best be interpreted as showing a series of overlapping epidemics caused by organisms with different resistance patterns and it seems clear that changes in these resistance patterns occurred from time to time.

Table 3. *Number of incidents due to* Shigella sonnei *colicine type* 3

		Antibiotic resistance pattern					
Sulphonamide		S	R	R	R	R	
Streptomycin		S	S	R	S	R	
Tetracycline		S	S	S	R	R	
Year	Quarter						
1956	4	.	.	.	.	.	
to		3	.	.	.	.	
1957	3	.	.	.	.	.	
	4	2	105	.	.	.	
1958	1	.	144	.	.	.	
	2	1	34	.	.	.	
	3	.	2	.	.	.	
	4	1	10	.	.	.	
1959	1–4	3	20	.	1	.	
1960	1–4	3	27	.	.	.	
1961	1–4	9	45	.	.	1	35 incidents in Kensington
1962	1–4	.	15	.	29	0	22 incidents in Camberwell
1963	1–4	.	48	3	1	2	47 incidents in Croydon
1964	1–4	1	.	.	.	.	
1965	1	0	0	0	0	1	

Table 4. *Number of incidents due to* Shigella sonnei *colicine type* 3A

		Antibiotic resistance pattern		
Sulphonamide		S	R	R
Tetracycline		S	S	R
Year	Quarter			
1957	3	.	1	.
	4	.	1	.
1958	1	.	6	.
	2	1	9	2
	3	2	4	1
	4	0	0	0

A single incident occurred in 1962.

The sequence of events following the introduction of colicine type 3 into the area was rather different and is shown in Table 3.

Five incidents due to sulphonamide sensitive strains were recorded during 1957, all of which occurred in the borough of Islington where the epidemic due to a

sulphonamide resistant strain started in the last quarter of 1957. This strain spread into the neighbouring borough of Stoke Newington but was only found elsewhere in very small numbers. The rapid and extensive change of antibiotic resistance pattern observed with types 7 and 13 was not found with this type and apart from localized outbreaks in 1961, 1962 and 1963 of twenty to fifty incidents in single boroughs it has remained an uncommon type.

A similar sequence of events can be seen with some of the less common colicine types. The single example of colicine type 3A must suffice, of which details are given in Table 4. After the end of 1958 a single isolation of type 3A was made in 1962 and two isolations in 1963. All three strains were resistant to sulphonamides but sensitive to streptomycin and tetracycline.

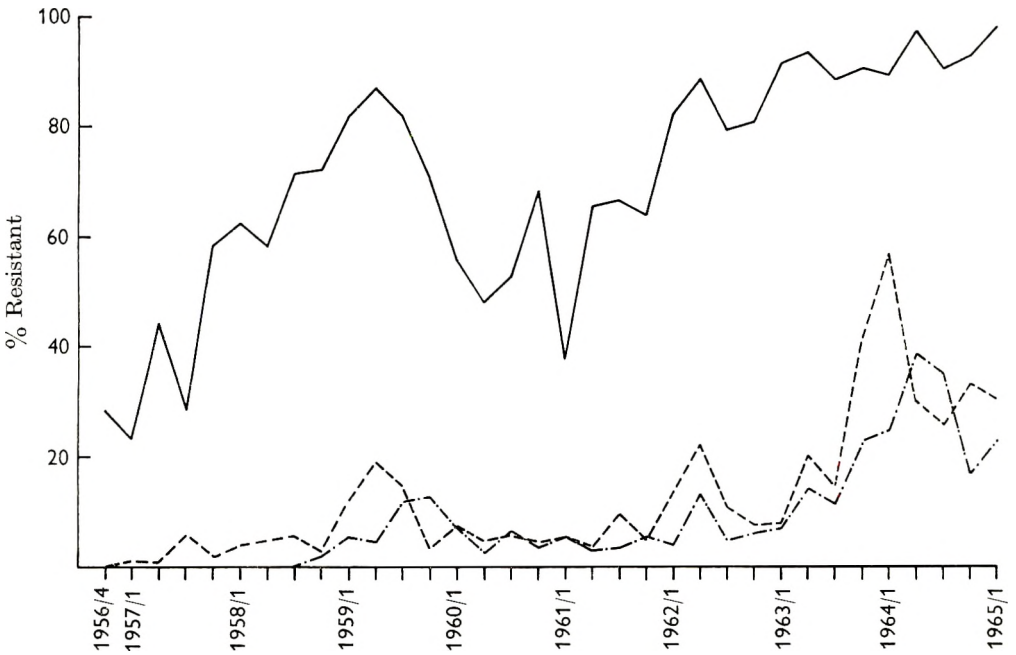


Fig. 3. Changes in sulphonamide and antibiotic resistance of *Sh. sonnei*, 1956–65. ——, Sulphonamide; - - -, tetracycline; — - — -, streptomycin.

It is of interest to see how the overall pattern of antibiotic resistance of *Sh. sonnei* has altered over the years. Details are given in figure 3.

It will be seen that, although the general tendency has been for *Sh. sonnei* to become more resistant to antibiotics this has not been an uninterrupted process. These fluctuations in resistance were related to the resistance patterns of the strain epidemic at any one time and it is clear comparing Fig. 1 with Fig. 3 that increased frequency of resistant strains was not related, except occasionally by chance, to an increased incidence of infection.

During the early part of this study, it seemed that strains of *Sh. sonnei* resistant to streptomycin or tetracyclines or to both drugs were causing for the most part

single incidents and spreading in the community only to a very limited extent, whereas some at least of the strains sensitive to these antibiotics spread widely in the same area. If this were so, the overall percentage of resistant strains would give an underestimate of the frequency with which these resistant strains arose in the community.

It is possible to obtain an estimate of the frequency with which antibiotic resistant strains arose in the community as follows; the number of colicine types recorded for any one borough in any one quarter (borough/quarter) varied between 0 and 9. Each occurrence of antibiotic resistant strains of different colicine type in any one borough/quarter might represent an organism 'newly' resistant whether it gave rise to one or to many incidents. The strains of *Sh. sonnei* from one borough/quarter might fall into 6 colicine types and there might be antibiotic resistant strains from 2 of these colicine types. In another borough/quarter there might be three colicine types one of which had antibiotic resistant representatives. Adding together the figures derived from the individual borough/quarters, the number of colicine types which had resistant representatives would represent the total of 'new' resistant strains, and the frequency of their occurrence can be compared with the frequency of antibiotic resistant strains in the overall totals. This has been done in Table 5. Since the frequency of antibiotic resistant strains has varied, the $8\frac{1}{2}$-year observation period has been divided into four intervals of approximately 2 years and the analysis confined to the fifteen boroughs from which large numbers of specimens have been regularly examined.

Table 5. *Comparison of the frequency of antibiotic resistant strains (a) of different colicine type in borough/quarters and (b) overall*

Period	No. of boroughs/ quarters	No. of colicine types	Percentage of colicine types with representatives resistant to			Total incidents	Percentage incidents due to strains resistant to		
			S	T	S and T		S	T	S and T
1956/4–1958/4	129	312	1·0	9·3	0	1888	0·4	3·1	0
1959/1–1960/4	120	424	18·7	18·0	2·4	2942	5·6	8·5	0·4
1961/1–1962/4	120	288	13·2	20·6	4·9	1328	6·2	10·2	1·6
1963/1–1965/1	131	349	34·7	46·5	18·0	2504	16·0	22·0	7·9

Note. Ten borough/quarters not examined. Doubly resistant strains have not been included under resistance to a single antibiotic. S = streptomycin; T = tetracycline.

It will be seen that the proportion of colicine types with antibiotic resistant strains in the borough/quarters was greater, by a factor of two- or three-fold, than the overall proportion of antibiotic resistant strains isolated at the same time. This method of estimating the frequency with which new resistant strains arose is clearly liable to errors due to the persistence of resistant strains in a borough from one quarter to the next and to the spread of a resistant strain over borough boundaries. There is evidence that both these occurrences have happened occasionally and these errors will make the figures in Table 5 for 'new' resistant strains an overestimate. On the other hand, no allowance has been made for the possibility

that a resistant strain of one colicine type might arise on more than one occasion in one borough/quarter; certainly, during the last 2 years when resistant strains have been fairly common, this must have occurred and will compensate to some extent for the other errors. It seems fair to conclude that, in general, strains of *Sh. sonnei* resistant to streptomycin and terramycin did not spread as widely in the general population as did sensitive strains. The fact that localized epidemics due to resistant strains occurred from time to time did not invalidate the general conclusion.

Factors responsible for colicine production can be transferred between members of the Enterobacteriaceae in the same way that resistance factors are transferred and it is not possible to believe that the colicine type of a strain of *Sh. sonnei* remains absolutely constant as it spreads through the community. Gillies (1964) has isolated strains of two colicine types from single individuals and observed a lack of uniformity of colicine type in a small proportion of household and institutional outbreaks which he attributed to mixed infections.

We have investigated 130 outbreaks of Sonne dysentery in day nurseries from which more than one strain of *Sh. sonnei* was examined in detail. All the strains of *Sh. sonnei* in 104 of these outbreaks proved to be identical in colicine type and resistance pattern. In ten outbreaks, strains of the same colicine type but different resistance pattern were observed, in eleven, strains of different colicine type but identical resistance pattern and in five, strains which differed both in colicine type and resistance pattern. Colicine type appeared just as likely to alter as antibiotic resistance.

It was possible to explain some of these discrepancies on the basis of multiple sources of infection but this became more difficult when, as has happened on several occasions, strains of *Sh. sonnei* of different colicine type but all possessing an unusual resistance pattern or other biochemical property were isolated from a day nursery. Similar observations have been made in day schools.

Table 6. *Comparison between the number of incidents and the number of different colicine types in a borough/quarter*

No. of incidents per borough/quarter	No. of borough/quarters	Mean no. of colicine types
0	37	
1–5	164	1·8
6–10	86	2·7
11–20	96	3·2
21–50	78	4·1
51+	39	5·3

Note. Ten borough/quarters not examined.

The number of different colicine types isolated from an area increased as the incidence of Sonne dysentery increased. Taking the borough/quarter as the unit of time and place and confining the analysis to the fifteen major boroughs, it will be seen from Table 6 that the mean number of colicine types detected in a borough/quarter increased as the number of incidents rose.

When large numbers of incidents occurred in a borough/quarter their distribution over the colicine types was not uniform. Usually most of the incidents were caused by one or two colicine types, the other types causing only a few incidents. Since epidemics of Sonne dysentery were often confined to a single borough or group of contiguous boroughs it is difficult to account for these few incidents, due to unexpected colicine types, except on the basis of change from the epidemic type.

Different colicine types appeared to behave differently in the population. Type 0 was present throughout the period under review in all boroughs and often gave rise to epidemics; strains with a particular resistance pattern might be present only for a limited time in a borough but the type as a whole persisted. On the other hand, strains of *Sh. sonnei* of colicine types 1A, 3, 3A, 4 and 13 produced local epidemics lasting 6–15 months in a borough, being virtually absent from that area before and after the epidemic. Intermediate between these two extremes was the behaviour of colicine types 2 and 7. The former, particularly during the early part of our period, tended to give rise to successive annual epidemics in some areas but only to single isolated epidemics in others. Type 7 when first introduced gave rise to local epidemics of finite duration but later caused epidemics going on over several years.

These impressions, that colicine types behaved differently, can be examined mathematically by determining the mean number of incidents per borough/quarter due to each colicine type of *Sh. sonnei*. The figures for the means were obtained by adding together the total number of incidents due to each colicine type and dividing by the number of borough/quarters in which that particular type occurred. Details are given in Table 7 which is confined to the fifteen major boroughs and has been subdivided into 4 periods of approximately 2 years.

Table 7. *Mean number of incidents per borough/quarter due to different colicine types*

	Colicine type								
Period	0	2	3	4	6	7	13	All other types	Total all types
1956/4–1958/4	7·0	6·7	15·7	2·9	2·0	6·1	.	1·8	5·4
	(102)	(82)	(19)	(32)	(27)	(16)	.	(33)	(311)
1959/1–1960/4	15·3	6·3	1·5	2·3	1·7	4·5	6·0	3·9	7·0
	(110)	(82)	(25)	(42)	(36)	(61)	(1)	(58)	(415)
1961/1–1962/4	6·5	2·7	4·9	2·9	1·7	6·0	10·6	1·6	4·6
	(85)	(36)	(19)	(31)	(29)	(65)	(3)	(22)	(290)
1963/1–1965/1	6·8	3·1	6·2	2·9	2·1	9·7	17·6	2·5	5·7
	(102)	(20)	(9)	(36)	(33)	(95)	(29)	(23)	(347)
Total	9·1	5·6	6·8	2·7	1·9	7·1	16·6	2·8	6·3
	(399)	(220)	(72)	(141)	(125)	(237)	(33)	(136)	(1363)

Figures in parentheses indicate the number of borough/quarters from which the relevant colicine type was isolated.

The mean number of incidents per borough/quarter due to a particular colicine type must give a measure of the ability of that particular type to spread in the population and cause overt infection. The figures could be vitiated by irregular

sampling of the population but the long observation period and the number of Local Authorities would seen to eliminate major error on that score.

It is impossible to avoid the conclusion that some strains of *Sh. sonnei*, e.g. types 0 and 7, were better able to spread in the population than other types, such as types 2, 4 and 6; there is also evidence to suggest that the ability of a colicine type to spread might vary with time and was not necessarily constant.

A similar analysis can be carried out to compare antibiotic resistant strains with sensitive strains of the same colicine type. This has been done in Table 8 for types 0 and 7—the only types for which sufficient resistant strains were available.

Table 8. *Mean number of incidents per borough/quarter due to sensitive and resistant strains of types* 0 *and* 7

		Colicine type 0				Colicine type 7			
	Streptomycin	S	R	S	R	S	R	S	R
	Tetracycline	S	S	R	R	S	S	R	R
Period (year and quarter)	1959/1–1960/4	13·0	2·1	4·6	1·0	4·4	1·7	1·2	1·0
		(109)	(31)	(44)	(4)	(56)	(16)	(8)	(1)
	1961/1–1962/4	5·7	2·7	1·8	1·3	5·8	1·4	1·5	2·0
		(77)	(15)	(27)	(8)	(62)	(5)	(11)	(1)
	1963/1–1965/1	5·5	1·5	2·4	3·1	5·4	4·3	3·1	1·8
		(88)	(30)	(42)	(20)	(74)	(30)	(49)	(18)
	Total	8·5	2·0	3·1	2·5	6·4	3·2	2·7	1·8
		(274)	(76)	(113)	(32)	(192)	(51)	(68)	(20)

Figures in parentheses represent the number of borough/quarters in which the appropriate strain appeared.

Table 9. *Drug resistance of colicine type* 6

	All strains except type 6				Strains of type 6			
Period (year and quarter)	Total strains	Sulphon-amide	Strepto-mycin	Tetra-cycline	Total strains	Sulphon-amide	Strepto-mycin	Tetra-cycline
1956/4–1958/4	2033	1158	7	66	58	7	0	0
		(57)	(0·3)	(3)		(12)		
1959/1–1960/4	3069	2025	181	273	67	12	1	4
		(66)	(6)	(9)		(18)	(1·5)	(6)
1961/1–1962/4	1406	1084	89	151	54	11	1	1
		(77)	(6)	(11)		(20)	(2)	(2)
1963/1–1965/1	2664	2497	448	594	68	31	(4	18
		(97)	(17)	(22)		(47)	(6)	(26)

Figures in parentheses are percentages.

Insufficient observations on resistant strains were available during the first $2\frac{1}{4}$ years but thereafter it seems clear that antibiotic resistant strains, on average, gave rise to fewer incidents in those borough/quarters in which they occurred than did sensitive strains.

The behaviour of colicine type 6 has been quite different from that of other colicine types; it has been isolated in small numbers from the whole area through-

out the whole of the observation period, yet the largest number of incidents due to this type in any one quarter has been 21. With one possible exception, it has never given rise to a local epidemic. It appears to be common in continental Europe (Abbott & Graham, 1961) and it has proved to be the type most commonly isolated from patients infected abroad. The sensitivity pattern of strains of colicine type 6 has differed from that of other types in that they were usually sensitive to sulphonamides and very few strains, until 1963, were resistant to streptomycin and the tetracyclines. Details are given in Table 9 from which it will be seen that strains of this colicine type are able to acquire resistance but have been slower to do so than the other types.

DISCUSSION

The most striking feature of this survey has been the appearance of new colicine types and new patterns of antibiotic resistance among strains of *Sh. sonnei* both in the area as a whole and in the individual boroughs. While, for example, it might be possible to account for the appearance of colicine type 13 in late 1962 on the basis of importation from outside, it is not possible on this basis to account for the fact that strains of this type with at least five different resistance patterns were isolated from north-east London during the subsequent 6 months. The fact that the number of colicine types isolated from an area increased as the frequency of Sonne dysentery increased in that area (Table 6) and that the incidents were not evenly distributed over the colicine types can most easily be explained on the basis that the more widespread the *Shigella* the greater will be the variety of strains of *E. coli* with which it comes in contact and the greater the opportunity for the acquisition of new colicine factors. This observation is in line with that of Gillies (1964) who found different colicine types in eight out of 480 households but in fifteen out of sixty-seven institutional infections. In the latter, a strain of *Sh. sonnei* would experience contact with a wider range of coliforms.

The changes in antibiotic resistance and colicine type observed over the $8\frac{1}{2}$ years can be best regarded as a result of the interaction and transfer of characters between *Sh. sonnei* and other intestinal organisms. The change from sensitive to resistant has been most marked with regard to sulphonamides, yet even here the change has not been constantly progressive. Davies (1954) selected strains from the area we examined in the same manner as ours and found 61 % of strains resistant to sulphonamide with a marked difference between different areas. This contrasts with our finding that 24 % of strains in the first quarter of 1957 were resistant, and shows that the fluctuations in resistance which can be seen in Fig. 2 certainly started before 1954. The proportion of strains of *Sh. sonnei* isolated from the population that are resistant or sensitive to a particular antibiotic merely reflects the resistance pattern of the current epidemic strain.

It seems inevitable that the proportion of strains resistant to streptomycin and tetracycline will increase. Antibiotic therapy—for dysentery or for some completely different condition—may allow a resistant strain of any member of the Enterobacteriaceae present in the gut in small numbers to become the dominant organism, to be passed to others and transfer its resistance to other organisms. At the same

time the human population is being continually infected with antibiotic resistant organisms derived from farm animals. Salmonellas derived from slaughter houses appear in the human population (McDonagh & Smith, 1958; Report, 1965) and the much more numerous *E. coli* derived from animals must do the same.

The epidemiology of Sonne dysentery in London as revealed by colicine typing has been characterized by local outbreaks, sometimes spreading into neighbouring areas, rather than generalized epidemics. A total of 286 incidents were recorded during the winter of 1957/8 due to type 3; all but 8 of these occurred in the adjacent boroughs of Islington, Stoke Newington and Shoreditch. During the epidemic due to type 13 which lasted for 2 years and was responsible for 560 incidents, only fifteen of these incidents occurred outside a group of contiguous boroughs in north-east London. Both of these episodes can be regarded as large local epidemics. Colicine type 7 gave rise to a generalized epidemic over the whole area during the winter of 1962/3 but when this strain first occurred during the summer of 1958, it was largely confined to a single area.

We have observed, on several occasions, the more or less simultaneous isolation of an unusual colicine type from a few incidents in two or more widely separated areas. In view of the failure of *Sh. sonnei* to spread widely from established local epidemics, it is difficult to account for this on the basis of a common source of infection. An alternative hypothesis would assume that the unusual colicine type originated *de novo* in several places at once. The widespread dissemination of a strain of *E. coli* with the appropriate colicine factors, e.g. in raw or cooked food, might allow local strains of *Sh. sonnei* to take on new colicine characters in several areas at the same time.

There is evidence that different strains of *Sh. sonnei* vary in their ability to spread widely through the population. These differences are shown in Table 7 for colicine type and in Tables 5 and 8 for strains resistant to streptomycin and tetracycline. These tables suggest that strains of colicine types 0, 7 and 13, sensitive to streptomycin and tetracycline, are more likely to spread widely than other strains. The power to spread widely did not seem to be possessed by all strains with these characters and was certainly not exclusive to them since sharp local episodes were caused, at one time or another, by strains of colicine type 1A, 2, 3, and 4.

Another phenomenon that requires explanation is the disappearance from the human population of strains of *Sh. sonnei* characterized by colicine type and antibiotic resistance. A small proportion of resistant organisms in a culture can be shown in the laboratory to have lost their resistance and to have reverted to sensitivity (Datta, 1962) but this seems unlikely to account for the disappearance of resistant strains from the human population. Strains of *Sh. sonnei* resistant to streptomycin and tetracycline often spread less widely than sensitive strains and might be expected to die out during the summer months, when Sonne dysentery is least common, simply because they failed to infect new individuals at the rate at which convalescent carriers ceased excreting. Such an explanation does not account for the disappearance of a strain such as the original epidemic type 13 sensitive to streptomycin and tetracycline. This strain, originally capable of spreading widely, spread through a population of about 1,000,000, gave rise to numerous incidents

and disappeared some 15 months after its original introduction. Many other similar, though less spectacular, episodes could be quoted.

It is tempting to ascribe to epidemic strains of *Sh. sonnei* a special 'ability to spread' that is not possessed by the majority of other strains and which is not related to any character at present detectable in the laboratory. This 'ability to spread' might merely be a power to outgrow the other organisms in the gut and to produce a higher concentration of *Sh. sonnei* in the faeces, but many other possibilities exist. It would be necessary to assume that this 'ability to spread' was an unstable character in order to account for the decline and disappearance of epidemic strains but some transferred characters may also be unstable. Certainly, some characters—antibiotic resistance and colicine production—can be transferred to *Sh. sonnei* in the laboratory and the evidence produced here suggests very strongly that this also happens in the human population. There is no reason why other characters which cannot yet be distinguished in the laboratory should not also be transferred to *Sh. sonnei*. Such an hypothesis which would ascribe part, at least, of the rise and fall of epidemics of Sonne dysentery to changes in the organism runs somewhat contrary to the teaching of classical epidemiology. The latter has not been particularly successful at explaining the spread of this infection so it is worth considering an alternative.

SUMMARY

The colicine type and antibiotic resistance have been determined on strains of *Sh. sonnei* derived from 9419 incidents of Sonne dysentery in London between October 1956 and March 1965.

The most striking observation has been the appearance of strains with patterns of colicine production and antibiotic resistance new to the area. These changes are best regarded as a result of the interaction and transfer of characters between *Sh. sonnei* and other intestinal organisms.

The general tendency has been for strains of *Sh. sonnei* to become increasingly resistant to antibiotics but this has not been an uninterrupted process. The proportion of drug-resistant strains at any one time depended on the properties of the current epidemic strain.

Spread of Sonne dysentery was essentially local, but some strains of *Sh. sonnei* were found to spread much more widely than others. Most, but not all, of the strains resistant to streptomycin and tetracycline possessed only limited powers of spreading.

REFERENCES

ABBOTT, J. D. & GRAHAM, J. M. (1961). Colicine typing of *Shigella sonnei*. *Mon. Bull. Minist. Hlth* **20**, 51.

ABBOTT, J. D. & SHANNON, R. (1958). A method of typing *Shigella sonnei* using colicine production as a marker. *J. clin. Path.* **11**, 71.

ANDERSON, E. S. & LEWIS, M. J. (1965). Characterization of a transfer factor associated with drug resistance in *Salmonella typhi-murium*. *Nature, Lond.* **208**, 843.

BRADLEY, W. H., RICHMOND, A. E., SHAW, C. H. & TAYLOR, I. (1958). Sonne dysentery in England and Wales. *Med. Offr* **99**, 175.

DATTA, N. (1962). Transmissible drug resistance in an epidemic strain of *Salmonella typhi-murium*. *J. Hyg., Camb.* **60**, 301.

DAVIES, J. R. (1954). A simple method of determining the sulphonamide sensitivity of *Shigella sonnei*. *Mon. Bull. Minist. Hlth* **13**, 114.

GILLIES, R. R. (1964). Colicine production as an epidemiological marker of *Shigella sonnei*. *J. Hyg., Camb.* **62**, 1.

HUTCHINSON, R. I. (1956). Some observations on the method of spread of Sonne dysentery. *Mon. Bull. Minist. Hlth* **15**, 110.

MCDONAGH, V. P. & SMITH, H. G. (1958). The significance of the abattoir in salmonella infection in Bradford. *J. Hyg., Camb.* **56**, 271.

OZEKI, H., STOCKER, B. A. D. & SMITH, S. M. (1962). Transmission of colicinogeny between strains of *Salmonella typhi-murium* grown together. *J. gen. Microbiol.* **28**, 671.

REPORT (1965). Salmonellae in cattle and their feedingstuffs and the relation to human infection. *J. Hyg., Camb.* **63**, 223.

WATANABE, T. (1963). Infective heredity of bacterial drug resistance. *Bact. Rev.* **27**, 87.

J. Hyg., Camb. (1966), **64**, 305
With 1 plate
Printed in Great Britain

A new colicine type (type 15) of *Shigella sonnei*

By R. R. GILLIES and D. O. BROWN
Bacteriology Department, University of Edinburgh

(*Received* 1 *February* 1966)

INTRODUCTION

Colicine typing of *Shigella sonnei* was introduced in this country by Abbott & Shannon (1958); the method depends on the production of various patterns of inhibition on a stock set of indicator or passive strains of other shigellas and interest in the technique has increased rapidly in the last few years. In an earlier publication (Gillies, 1964) modifications of the original technique were described and further evidence was offered of the reliability of colicine type as an epidemiological marker; a hitherto unrecognized type (type 14) of *S. sonnei* was described and evidence was given of its distinctive character.

The present paper furnishes evidence for the existence of another new colicine type (type 15) of *S. sonnei* which was first recognized in Edinburgh in 1965.

MATERIALS AND METHODS

Strains

The twenty-two cultures, from which 181 colonies of the new colicine type of *S. sonnei* were tested, were harvested from faecal specimens in laboratories in Edinburgh and Glasgow and all but three were from residents in Edinburgh. Each colony was identified by its biochemical reactions in tests with composite media (Gillies, 1956) and by agglutination tests with specific antiserum.

The indicator strains were those employed by all British workers and were acquired originally from Dr J. D. Abbott.

Media

Infusion broth was used to culture the indicator strains once weekly or as often as required and was prepared according to Cruickshank (1965); tryptone soya agar (T.S.A.) (Oxoid) was prepared according to the maker's instructions and 5% horse blood added (T.S.B.A.).

Typing technique

This has been detailed previously (Gillies, 1964) and may be summarized as follows. The strain of *S. sonnei* to be typed is inoculated in a diametric streak on a T.S.B.A. plate which is then incubated at 35–36° C. for 24 hr. The macroscopic growth is removed with a sellotape-sheathed glass slide and microscopic remnants of growth are sterilized by pouring 3–5 ml. of $CHCl_3$ into the lid of the Petri dish and replacing the medium-containing portion over the lid; after 15 min. the plate is opened and residual $CHCl_3$ is decanted into a beaker. The medium is exposed to

the air for 3 min. and the fifteen indicator strains are then applied at right-angles to the original line of growth.

The plate is then re-incubated overnight at 37° C. and during this period any colicines produced by the original inoculum will exert their inhibitory activity on the indicator strains. The various patterns of inhibition are then observed (Table 1).

RESULTS

A profuse and almost pure culture of *S. sonnei* was isolated from a faecal specimen submitted from a 7-year old boy on 17 October 1965. Colicine typing of three colonies from the diagnostic plate gave identical patterns of inhibition on the indicator strains (Plate 1), but these differed from the patterns given by any of the sixteen recognized colicine types (Table 1). Fifteen more colonies from the same diagnostic plate were tested and they also gave this new pattern of inhibition; this child was one of a family (S) of eleven and in the next few days five siblings and the father were found to be excreting *S. sonnei*.

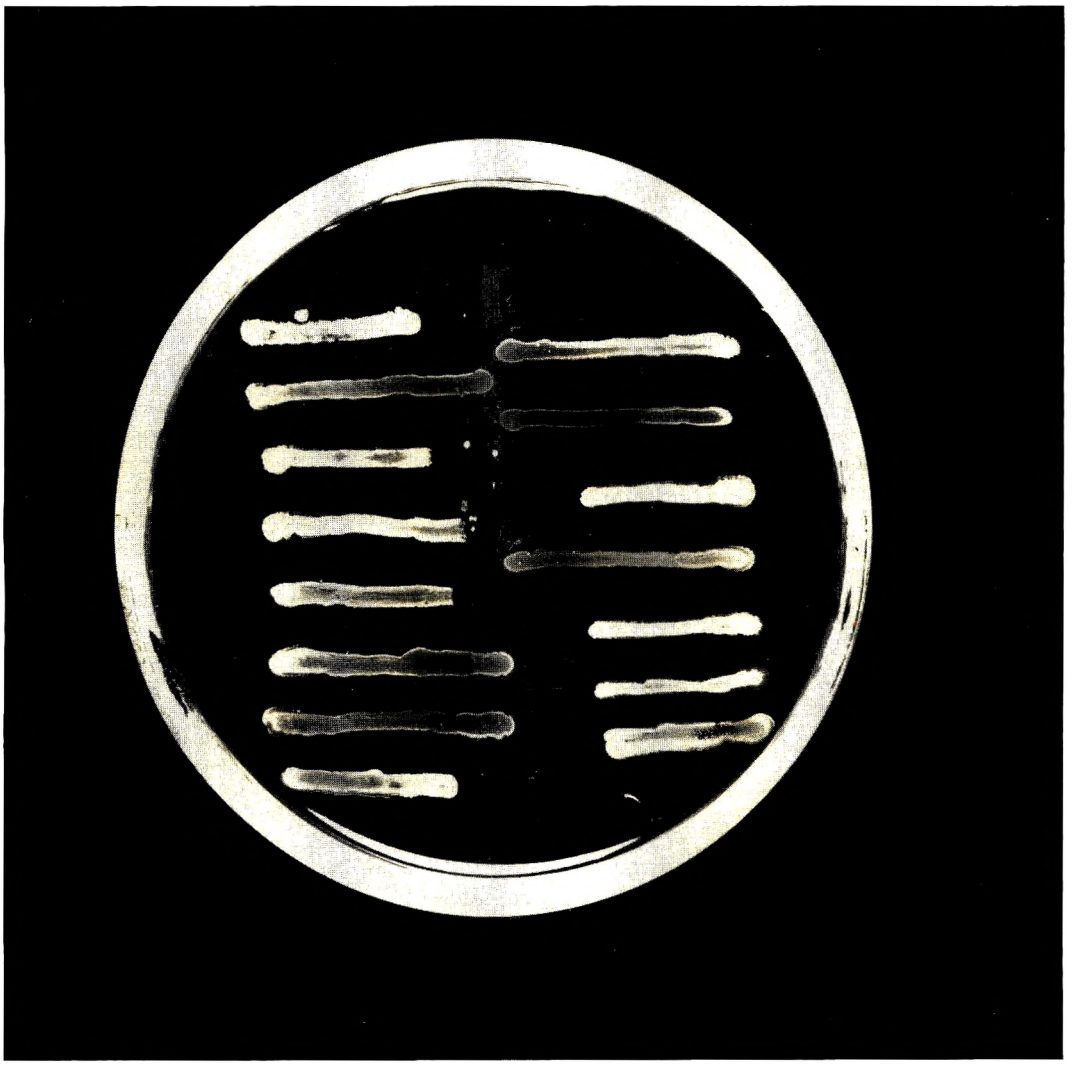
Table 1. *Patterns of inhibition given by seventeen colicine-type (producer) strains of* Shigella sonnei *in tests against fifteen standard indicator strains*

Indicator strain no.	Colicine type of producer strain																
	1*a*	1*b*	2	3	3*a*	4	5	6	7	8	9	10	11	12	13	14	15
1	+	+	−	+	+	+	+	−	−	+	+	+	−	+	−	+	+
2	+	+	+	+	+	+	+	+	−	−	+	+	−	+	+	+	−
3	+	+	+	+	+	+	+	−	+	+	+	+	−	−	+	+	+
4	−	−	−	−	−	v	v	−	−	−	−	−	−	−	−	v	+
5	−	−	−	+	+	+	+	−	−	+	v	+	−	+	−	+	+
6	+	+	−	+	+	−	+	+	−	−	+	+	−	+	+	+	−
7	+	+	−	+	+	−	+	+	−	−	+	+	−	+	+	+	−
8	−	−	−	+	+	+	+	−	−	+	+	+	−	+	−	+	+
9	−	+	+	+	+	+	+	+	−	−	−	+	−	+	+	+	−
10	+	+	−	+	−	−	+	−	−	−	−	+	−	−	−	−	−
11	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	+	+
12	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−
13	−	−	−	+	+	+	+	−	−	+	+	+	−	+	−	+	+
14	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+
15	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+

The indicator strain numbers correspond respectively with: *S. sonnei* 2, 56, 17, 2 M, 38, 56/56, 56/98, R 1, R 6; *S. schmitzi* M. 19 (NCTC 8218); *S. sonnei* 2/7, 2/64, 2/15, R 5 and *Escherichia coli* Row.

+ = Inhibition of an indicator strain; v = variable reaction; − = no inhibition of an indicator strain.

Typing of several colonies from each of the diagnostic plates revealed that every strain gave the new inhibitory pattern. During this time another family (L) submitted specimens of faeces and the strains of *S. sonnei* isolated from the index case and the other four members of this household gave the inhibition pattern of the new colicine type.



R. R. GILLIES AND D. O. BROWN

(*Facing p.* 306)

Inquiry at the Public Health Department revealed that although the two families lived in different municipal wards they regularly visited each other's homes.

DISCUSSION

Our indices of the reliability of colicine type as an epidemiological marker of *Shigella sonnei* are that replicate isolations from an individual should all be of the same type and that there should be uniformity of type in all cases in an epidemic situation; these indices are fulfilled completely in the material presented here.

The number of specimens, the number of positive isolations and the number of colonies typed are summarized in Table 2.

Table 2. *Summary of epidemiological information concerning colicine type* 15 *strains of* Shigella sonnei

	Family S	Family L	Family B	Individuals
No. of persons in family or group	11	5	2	4
No. of specimens	22	8	2	4
No. of persons with *S. sonnei* (type 15) in 1st specimen	7	5	2	4
No. of persons with *S. sonnei* (type 15) in 2nd specimen	4	0	N.T.	N.T.
No. of positive specimens = 22				
No. of colonies tested = 181				
Average number of colonies tested per specimen = 9 (range 1–33)				

N.T. = not tested.

Search of our records since 1959 revealed two further, unrelated patients who were excreting strains of *S. sonnei* that gave inhibition patterns identical with those of the strains from the two families, S and L. These isolations had been made in October 1963 and March 1965 and the strains had been labelled 'unclassifiable' since the pattern was then new and we had no evidence of its significance. The child from whom the isolation was made in March 1965 was, at the time, living in a residential nursery. The possibility was considered that some of the children in family S may have been accommodated in the same nursery at that time, but this was not so.

Similarly no link could be found between families S and L and the adult case in 1963. Eight days after the last isolation from family S a child with bacillary dysentery in another residential nursery was found to be excreting *S. sonnei* of the new colicine type, but no association could be found with any of the previous cases.

The latter child had only recently come from Glasgow and this fact prompted the colicine typing of strains of *S. sonnei* acquired from the City Laboratory in Glasgow. Of 584 strains thus examined three were of the new type; two of these strains had been isolated during August, 1965 from a husband and wife (family B) and the third strain from an unrelated case in November 1965. Search of our records of colicine examinations made on strains received from Glasgow laboratories in previous years did not reveal any instances of the new type.

We considered the possibility that the new type pattern might be an artifact associated with some disturbance of the indicator strains, either by contamination or through variation in their sensitivity to the various colicines. This explanation was unlikely since other producer strains that were being typed at the same time as those of the new type gave various patterns characteristic of established types. Nevertheless, we tested several strains of each established type from our stock of cultures and each gave its typical pattern of inhibition (Table 1).

It will be interesting to note the spread of this new type (type 15) in our locality and we hope that, following this report, its recognition elsewhere will be communicated.

SUMMARY

1. A new colicine type (type 15) of *Shigella sonnei* is described.
2. The epidemiological circumstances associated with its appearance in Edinburgh and Glasgow are summarized.
3. Our indices of reliability as an epidemiological marker are fulfilled by this new type.

This work was supported by Public Health Service Grant AI 04833-03 from the National Institute of Allergy and Infectious Diseases; we are indebted to Dr J. C. M. Sharp of the Edinburgh Public Health Department for providing much epidemiological information and to Dr T. F. Elias-Jones, Director of the City Laboratory, Glasgow for supplying strains from that city.

REFERENCES

ABBOTT, J. D. & SHANNON, R. (1958). A method of typing *Shigella sonnei* using colicine production as a marker. *J. clin. Path.* **11**, 71–7.

CRUICKSHANK, R. (1965). *Medical Microbiology*, 11th ed. p. 740. Edinburgh and London: E. and S. Livingstone Ltd.

GILLIES, R. R. (1956). An evaluation of two composite media for preliminary identification of *Shigella* and *Salmonella*. *J. clin. Path.* **9**, 368–71.

GILLIES, R. R. (1964). Colicine production as an epidemiological marker of *Shigella sonnei*. *J. Hyg., Camb.* **62**, 1–9.

J. Hyg., Camb. (1966), **64**, 309
Printed in Great Britain

The isolation and classification of Tern virus: Influenza Virus A/Tern/South Africa/1961

By W. B. BECKER

C.S.I.R. and U.C.T. Virus Research Unit, University of Cape Town, South Africa

(*Received* 31 *December* 1965)

THE EPIZOOTIC IN COMMON TERNS—*STERNA HIRUNDO*

The field observations on the epizootic among Common Terns along the coast of the Cape Province of South Africa in April 1961 have been reported in detail by Rowan (1962). The Common Tern is usually seen in the Republic of South Africa from October to February, but in 1961 many Common Terns delayed their migration to the breeding grounds in the temperate regions of the Northern Hemisphere until May. Several dead terns were reported in the region of Cape Town in the second and third weeks of April, but the epizootic became explosive in the third and fourth weeks and either spread rapidly or was multifocal in origin along the 1000-mile stretch of coast from Port Elizabeth to Lambert's Bay (Fig. 1). The

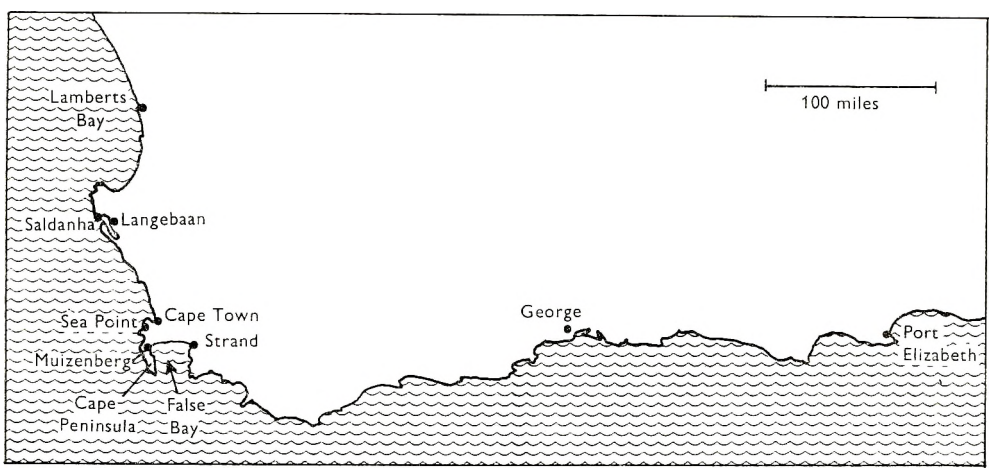


Fig. 1. Places in South Africa where dead Common Terns were found during the epizootic of 1961.

mortality was high as evidenced by the finding of 1300 dead terns in four small areas alone. Only Common Terns were affected; no overt infection of any other species was noted. No more dead terns were found after the second week in May, by which time most of the remaining birds had left, presumably on migration northward.

The causal agent was isolated from a number of afflicted terns and was named Tern virus (Becker, 1963).

MATERIALS AND METHODS

Virus strains

Tern virus was classified as a result of this work as influenza virus A/Tern/South Africa/1961. Influenza virus A/Chicken/Scotland/1959 (Chicken/Scot. virus) was obtained from Dr J. E. Wilson.* Other viruses used were influenza virus A_2/Cape Town/51/1961 (Influenza A_2), influenza virus B/Lee (Influenza B), influenza virus C/JJ/1950 (Influenza C) and Newcastle disease virus strain Komarov (NDV).

Virus propagation

The virus strains were propagated by inoculating 10^3 to 10^5 egg-infective doses (EID)50 allantoically, or amniotically in the case of Influenza C, into several embryonated hens' eggs and subsequently harvesting and pooling the infected fluids which contained between 10^7 and 10^9 EID 50 of virus per ml. Some strains were also propagated in chick embryo cell monolayer cultures. Reference stocks were lyophilized and working stocks were stored in ampoules at $-20°$ C.

Embryonated hens' eggs

Leghorn–Australorpe-cross eggs from a commercial source were used and incubated at 36–37° C. after inoculation.

White mice

The highly inbred laboratory strain of white mice was used.

Tissue cultures

Monkey kidney (MK) cells. Standard methods were used to prepare secondary roller-tube cultures of Vervet monkey (*Cercopithecus aethiops pygerythrus*) kidney epithelial cells. The growth medium consisted of Hanks's salt solution containing 0·5 % lactalbumin hydrolysate, 5–10 % filtered calf serum, 0·002 % phenol red, 0·035 % sodium bicarbonate and antibiotics. The maintenance medium contained 0·135 % sodium bicarbonate and 0·5 % fowl serum in place of calf serum.

Strain KB malignant human epithelial cell line (KB). KB cell roller-tube cultures were prepared according to standard methods. The MK cell media were used except that the content of calf serum was 10 % in the growth medium and 10 % of fowl serum was used in the maintenance medium.

Chick embryo (CE) cells. Monolayers of CE cells were cultured in Petri dishes by the method of Porterfield (1960) based on the technique of Dulbecco (1952). Roller-tube cultures were prepared by seeding with approximately 10^6 cells per ml. of growth medium which was also used as maintenance medium.

All tissue cultures were incubated at 36–37° C.

Fixation and staining of cells grown on cover-slips. Cell sheets grown on flying cover-slips in roller tubes were fixed in absolute alcohol or in Bouin's fluid and stained with haematoxylin and eosin following stardard procedures.

* Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food, Eskgrove, Lasswade, Midlothian, Great Britain.

Haemagglutination (HA) and haemagglutination-inhibition (HI) tests

HA and HI tests were done by standard methods on Perspex plates (W.H.O., 1959).

Species *Sea-birds*

Virus studies were carried out on three affected Common Terns submitted during the epizootic; on thirteen sea-birds collected in 1961 after the epizootic had ended, of which three were Common Terns, three Arctic Terns (*Sterna macrura*), four Swift Terns (*Sterna bergii*), two Cape Cormorants (*Phalacrocorax capensis*) and one a Hartlaub's Gull (*Larus hartlaubii*); and on a further three Common Terns received in 1963.

Autopsy

Live birds were exsanguinated and blood was obtained from the opened heart of dead birds. Using aseptic techniques, the liver, spleen, heart, lungs, kidneys and brain were removed. Each organ except the brain was washed in three 20 ml. lots of sterile saline, a sample was removed for viral studies and the remainder fixed in 10 % formol-saline for histological examination.

Preparation of tissue emulsions

Serum was separated and stored at − 20° C. The blood clots and tissue samples were emulsified (10 %, w/v) in MK cell maintenance medium without serum. The emulsions were frozen and thawed twice, clarified by centrifugation and stored at − 20° C. or in a dry-ice cabinet.

Virus titration

Serial tenfold dilutions of suspensions were inoculated allantoically in 0·1 ml. amounts into eggs, four or six per dilution, which were examined after 48–72 hr. for death of the embryo and a positive HA test on the embryonic fluids. Alternatively CE cell cultures were employed and examined for cytopathic effect (CPE) daily for 7–14 days. The infective dose 50 % end-point (ID 50) was calculated according to the method of Reed & Muench (1938).

Neutralization tests

The standard test utilizing a constant dose of virus was carried out in CE cell roller tubes. The titre of a serum was expressed as the reciprocal of the highest dilution which neutralized the virus in at least 50 % of the inoculated cultures.

Complement-fixation (CF) tests

Both the small-volume method in tubes and the micro-method on standard Perspex HA plates were used following a procedure similar to that described in the W.H.O. Technical Report Series (1959). Overnight incubation at 4° C. was employed. The diluent was that of Mayer, Osler, Bier & Heidelberger (1946). Complement was preserved by the method of Richardson (1941) and 2 M.H.D. were used in the test.

Complement-fixing antigens

The procedures followed for the preparation of the type-specific nucleoprotein (NP) antigen and the strain specific haemagglutinin (V) antigen were based on the methods of Hoyle (1952) as modified by Lief & Henle (1956*a*, *b*) and Fabiyi, Lief & Henle (1958). Influenza C virus was however concentrated and partially purified by differential centrifugation, treated with ether and the resultant mixture of nucleoprotein and haemagglutinin used as antigen.

Sera prepared

Anti-V sera. Strain-specific sera were prepared according to the method of Fabiyi *et al.* (1958).

'Anti-NP' sera. These sera were prepared using the method of Lief, Fabiyi & Henle (1958) except that the same strain was employed for the initial intranasal inoculation and as the source of the NP for the second inoculation. Consequently, the guinea-pig immune sera were expected to have both anti-NP and anti-V antibodies. In the case of Influenza C the second inoculum consisted of a mixture of V and NP antigens.

Immune ferret sera. The animals were bled out 3–4 weeks after intranasal infection.

Immune chicken sera were obtained from birds (Leghorn–Australorpe cross) convalescing from experimental infection with Tern or Chicken/Scot. virus.

Immune rabbit sera. Rabbits were immunized with a series of four inocula of Tern-virus-infected allantoic fluid administered by the intramuscular (IM), intraperitoneal or intravenous routes over a period of 5 weeks.

Sera acquired

Anti-Fowl-plague virus: strain Brescia, strain Alexandrien and strain 'N'—Plum Island Animal Disease Laboratory. Chicken/Scot. virus immune chicken serum—Dr J. E. Wilson. NDV hyperimmune chicken sera—Onderstepoort Veterinary Laboratory. Human convalescent Mumps sera—C.S.I.R. and U.C.T. Virus Research Unit. Influenza A_2/Asia/57 and B/Johannesburg/33/58 hyperimmune rabbit serum —N.V. Philips-Duphar. Influenza A/Phil./53, A/FM1/47, A/PR8/34, Mumps virus, Para-influenza 1, 2 and 3 hyperimmune guinea-pig sera; Simian myxovirus (SV5) hyperimmune rabbit serum—Microbiological Associates.

RESULTS

Virological investigation of sea-birds

Isolation of Tern virus

Tern virus was isolated from three Common Terns (birds 1, 2 and 3, Table 1), which were the only birds received during the epizootic. Two pools were prepared from the organ emulsions of each bird by mixing equal portions of brain and liver, and of heart, lung and kidney emulsions. The pools were inoculated in 0·1 ml. amounts into embryonated eggs allantoically, into both KB and MK cells and into newborn mice intracerebrally.

In the case of bird 1, the embryos of the inoculated eggs died within 48 hr. and presented a rather striking appearance; their external surfaces were congested and showed punctate and frequently large focal haemorrhages. The allantoic fluids harvested from these eggs gave positive HA tests with chicken red cells and contained 80 haemagglutinating units (HAU) per ml. while HA tests on control allantoic fluids were negative. All fluids were bacteriologically sterile. The haemagglutinating agent could be passed serially and showed enzymatic activity similar

Table 1. *Virological data on four of the sea-birds from the Cape Peninsula region*

Bird no....	1*	2*	3*	4†
Species	Common Tern	Common Tern	Common Tern	Hartlaub's Gull
Received	27. iv. 61	27. iv. 61	11. v. 61	6. ix. 61
Condition	Dead 2 days	Dead 2 days	Ill	Alive
Autopsy	27. iv. 61	27. iv. 61	11. v. 61	14. ix. 61
Storage before titration	72 days, −20° C.	78 days, −20° C.	5 days, −70° C.	1 day, −20° C.
Tern virus content‡				
Heart	4·3	1·9	Trace	3·75
Lung	5·1	3·3	Trace	3·5
Liver	3·7	Trace	Trace	5·25
Kidney	3·5	1·7	Trace	4·75
Brain	1·7	–	–	–
Blood	5·1	3·7	–	NT
Serum HI antibody titre	NT	NT	< 5	< 5

* Naturally infected.
† Experimentally infected.
‡ $\log_{10}$EID 50 per g. of tissue.
–, No virus isolated. NT, Not tested.

to that of the influenza viruses. The conclusion that it was a myxovirus was confirmed by subsequent serological and morphological studies and it was named Tern virus. Pass 1 in KB cells was subinoculated after 9 days into fresh KB cells and 7 days thereafter CPE was noted. A second subinoculation resulted in CPE in 6 days and the culture fluid contained 1280 HAU per ml. while control fluids showed no haemagglutinating activity. The results of culture in MK cells and newborn mice were negative.

A similar agent was isolated from birds 2 and 3 in eggs, but not in KB or MK cells nor in newborn mice.

Prototype strain of Tern virus

The strain isolated in eggs from the heart-lung-kidney pool of bird 1 was employed as the prototype strain of Tern virus. HI tests using immune serum prepared against this strain showed that the same virus was isolated from all three birds.

Growth of Tern virus in embryonated eggs

The embryonated egg could be infected via all routes of inoculation with Tern virus, which could be recovered in approximately equal concentrations from all

the tissues of the embryo. The allantoic route was routinely used and the allantoic and amniotic fluids harvested as the most suitable sources of virus. These fluids usually contained $10^{7.5-9}$ EID 50 of Tern virus/ml.

End-points were sharp in titrations in eggs and the results were read within 48–72 hr. of inoculation.

Birds 1 and 2 were received dead. Bird 3 was received alive but ill and sat huddled up with ruffled feathers and was unwilling to move when disturbed. It is noteworthy that the virus content of some of the organs of Tern 1 was moderately high despite the storage of the emulsions at $-20°$ C. for 72 days. Less virus was found in the tissues of Tern 2, and only traces in those of Tern 3 in the serum of which no HI antibody to Tern virus was detected.

Post-epizootic period

In the post-epizootic period the only evidence of natural infection with Tern virus was the presence of specific HI and neutralizing antibodies in the serum of each of two Common Terns shot in July 1961. Two Swift Terns inoculated experimentally with live Tern virus showed no ill effects but developed HI and neutralizing antibodies in their sera.

Bird 4 (Table 1), a Hartlaub's Gull, died on the third day after IM inoculation of Tern virus. Virus was present in moderately high concentration in the tissues.

Histological examination of the sea-birds

Lesions were only found in birds 1, 2 and 3 (Table 1), which had a meningoencephalitis; no histological lesions were detected in the remaining sixteen birds, including those inoculated experimentally (C. J. Uys, personal communication).

Serological classification of Tern virus

Non-specific inhibitors

No non-specific inhibitors of Tern virus haemagglutination were detected in the following sera which were heated at 56° C. for 30 min. and tested at an initial dilution of 1/5 (in the case of birds) or 1/10 against 4 HAU of unheated Tern virus: sera of four Swift Terns, a Common Tern, an Arctic Tern, a Hartlaub's Gull, three ferrets and five rabbits; pooled and individual guinea-pig and chicken sera, pooled human, pooled mouse and pooled rat sera, calf and horse sera.

HI tests

Tern virus haemagglutination was inhibited by homologous and Chicken/Scot. virus immune serum (Table 2). No inhibition occurred with any of the other sera listed under materials and methods.

Cross-neutralization tests in CE cells

The only demonstrable cross-neutralization was between Tern and Chicken/Scot. viruses (Table 3), which showed a close reciprocal antigenic relationship (Table 4).

Table 2. *HI tests with Tern virus antigen and myxovirus strain-specific sera*

Strain-specific serum	Source of serum	HI antibody titre against 4 HAU of Tern virus	Homologous HI antibody titre
Tern/SA/1961	Rabbit	160	160
Chicken/Scot./1959*	Chicken	160	640
Fowl plague			
(1) Strain Brescia	–	< 4	–
(2) Strain Alexandrien	–	< 4	–
(3) Strain N	–	< 4	–
NDV (Komarov)	Chicken	< 5	160

* Serum received from Dr J. E. Wilson.
–, Information not available.

Table 3. *Cross-neutralization tests in chick embryo cell cultures*

Strain-specific serum	Source	Dilution used	Neutralizing antibodies against: Tern virus	Chicken/Scot. virus	NDV	Homologous HI antibody titre
Tern/SA/1961	Rabbit	1/5	+	+	–	160
Chicken/Scot./1959*	Chicken	1/10	+	+	–	640
Fowl plague						
(1) Strain Brescia	No data	1/5	–	–	–	
(2) Strain Alexandrien	No data	1/5	–	–	–	
(3) Strain N	No data	1/5	–	–	–	
NDV (Komarov)	Chicken	1/5	–	–	+	160

+, Antibodies present. –, No antibodies detected.
* Serum received from Dr J. E. Wilson.

Table 4. *Cross-neutralization tests in chick embryo cell cultures*

Serum of chicken convalescent from infection with	Neutralizing antibody titre against: Tern virus (100 ID 50)	Chicken/Scot. virus (50 ID 50)
Tern virus	320	320
Tern virus	1280	320
Chicken/Scot. virus	160	2560
Chicken/Scot. virus	1280	5120
Control chicken	< 5	< 5

CF tests

Several batches of antigens and immune guinea-pig sera were prepared for Tern, Chicken/Scot., and Influenza A_2, B and C viruses. The results of cross-CF tests (Table 5) indicated the similarity of the nucleoprotein of Tern, Chicken/Scot. and Influenza A_2 viruses. The strain-specific antigens of Tern and Chicken/Scot. viruses were closely related to each other but not to the Influenza A_2 antigen. There was

no antigenic relationship between Influenza B and C viruses or between these two viruses and Tern, Chicken or Influenza A_2 viruses.

Guinea-pigs immunized with purified Tern virus V antigen and shown to produce strain-specific CF antibodies also developed homologous HI and neutralizing antibodies (Table 6).

Growth in CE cells

Tern and Chicken/Scot. viruses produced CPE in CE cells with the formation of intranuclear inclusions, a feature which has apparently not been recorded before in Influenza A strains. Both strains produced plaques in CE cell monolayers in Petri dishes.

Table 5. *Cross-CF tests*

Virus nucleoprotein antigen	'Anti-NP' sera				
	Tern	Chicken/Scot.	Influenza A_2	Influenza B	Influenza C*
Tern	1024†	256	512	< 8	< 8
Chicken/Scot.	256	256	512	< 8	< 8
Influenza A_2	128	128	512	< 8	< 8
Influenza B	< 8	< 8	< 8	256	< 8
Influenza C*	< 8	< 8	< 8	< 8	256
Virus strain-specific antigen	**Strain-specific sera**				
	Tern	Chicken/Scot.	Influenza A_2	Influenza B	Influenza C*
Tern	256	64	< 8	< 8	< 8
Chicken/Scot.	256	256	< 8	< 8	< 8
Influenza A_2	< 8	< 8	256	< 8	< 8
Influenza B	< 8	< 8	< 8	512	< 8
Influenza C*	< 8	< 8	< 8	< 8	256

* Influenza C antigen consisted of ether-treated purified virus which was also used to prepare the immune sera.

† All tests were two dimensional. Figures represent the reciprocal of the highest serum dilution giving > 50 % fixation with any of the antigen dilutions tested.

Table 6. *Titration of homologous CF, HI and neutralizing antibodies in the sera of guinea-pigs immunized with purified strain-specific Tern virus antigen*

Strain-specific guinea-pig sera	Serum antibody titres against Tern virus antigen		
	CF	HI	Neutralizing
1	128	80	2560
2	128	40	1280
3	256	160	2560

DISCUSSION

Classification of Tern virus

Serological relationship

Tern virus is an avian strain of influenza A, classified as Myxovirus influenzae A/Tern/South Africa/1961.

Its haemagglutinating properties were similar to those of the influenza viruses.

Serological studies showed that it had type-specific nucleoprotein antigens similar to the Influenza A2 strain with which it was compared. However, the strain-specific haemagglutinin of Tern virus was antigenically quite distinct from that of other influenza strains investigated with the single exception of Chicken/Scot. virus which had an antigenically closely related haemagglutinin. This relationship was confirmed by H. G. Pereira (personal communication), and Pereira, Tumova & Law (1965) who also found no antigenic sharing between Tern virus haemagglutinin and the haemagglutinins of the following viruses: Fowl plague, Duck/England/1956 and 1962, Duck/Czechoslovakia/1956, Turkey/England/1963 and a virus recently isolated from turkeys in Canada.

Electron microscopy

Electron microscopical studies of Tern and Chicken/Scot. viruses (Becker, 1963 1964*a*, *b*) showed that both strains had the same structure as other influenza viruses which have been discussed by Waterson, Hurrell & Jensen (1962). The study of the morphology of Tern virus, however, did raise several new points: in high resolution electron micrographs of ether-fractionated, purified haemagglutinin the radiating rods of haemagglutinin appeared to be delicate tube-like structures linked at their central ends to form the viral envelope; both in ether-fractionated preparations of nucleoprotein and in ultra-thin sections of whole virus particles the nucleoprotein was clearly shown to be a double helix which was enantiomorphous; the double helix appeared to be regularly arranged within the viral particle and to lie adjacent to the viral envelope where, under certain conditions, it simulated a membrane.

Comparison of Tern and Chicken/Scot. viruses

A comparison of Tern and Chicken/Scot. viruses (to be published) has shown that they have many features in common and are probably variants of the same strain.

Epizootiology of Tern virus infection

Tern virus as the causal agent of the Tern epizootic

Tern virus was isolated from all three diseased Common Terns (birds 1, 2 and 3, Table 1) collected during the epizootic, and the same virus was isolated from a further two diseased Common Terns examined at the Onderstepoort Veterinary Laboratories (R. A. Alexander, personal communication). In birds 1 and 2 the virus content of the organs was high considering the long interval of storage at $-20°$ C. before titration. Bird 3 was probably examined at an early stage of infection, before the virus had had sufficient time to establish itself. In addition, serum was obtained from each of two Common Terns collected shortly after the epizootic and both sera contained HI and neutralizing antibodies to Tern virus, providing presumptive evidence that they had recovered from infection with Tern virus. Unfortunately it was not possible to capture live Common Terns but experimentally inoculated chickens developed an acute illness similar to that noted in Terns (Becker, 1964*b*).

It seems reasonable to conclude that during the epizootic there was widespread

infection of Common Terns with Tern virus. It is interesting that two captive Swift Terns which are not migrants, were clinically unaffected by experimental inoculation with live Tern virus, but antibody formation was stimulated. The other bird inoculated experimentally was the gull (bird 4, Table 1) which was not doing well in captivity and died 3 days after IM inoculation of Tern virus. It is not clear if the gull died of Tern virus infection; no histological lesions were found despite the isolation of virus from the tissues.

Sea-birds as a source of infection of domestic poultry

The isolation of Tern virus raises interesting epidemiological possibilities. The outbreak in chickens in Scotland caused by Chicken/Scot. virus preceded the Tern epizootic by about 17 months and occurred during stormy weather which drove sea-birds a little inland to take shelter. Large numbers of Herring Gulls (*Larus argentatus*) were at that time working the farm at which the outbreak in chickens occurred in November 1959 (J. E. Wilson, personal communication). The chickens might have contracted the infection from sea-birds, a viewpoint possibly supported by the preceding mass mortality in Kittiwakes (*Rissa tridactyla*) and Fulmars (*Fulmaris glacialis*) from February to August 1959 (Joensen, 1959) off the coast of Britain and Scandinavia. Unfortunately the aetiology of the last-mentioned outbreak was not investigated, but it is tempting to think it was caused by the Tern virus which was isolated at Cape Town some 18 months later in 1961, from migrant European Common Terns.

One might postulate: that certain sea-birds suffer latent or sporadic infection with avian influenza; that epizootics may be precipitated in them by conditions of stress, e.g. poor feeding under unfavourable weather conditions such as preceded the Tern epizootic; and that spread to other sea-birds or domestic poultry may occur.

Of particular interest is the investigation of Blaxland (1951) into the possible role of sea-birds in the spread of NDV amongst domestic poultry in Scotland, neighbouring off-shore islands and Ireland. NDV was isolated from the bone marrow emulsions of six out of twenty-six shags (*Phalacrocorax aristotelis*) and antibodies to NDV were found in eighteen of fifty-four blood samples of shags and cormorants (*P. carbo*). Blaxland considered sea-birds as a possible means of spread of NDV because of the local habit of shooting shags for the table and throwing the offal to the poultry. Wilson (1950) isolated NDV from a gannet (*Sula bassana*) caught on the Orkney islands. Wells (1963) described an outbreak of fowl plague on a turkey farm on the Norfolk coast and concluded that the infection may have been introduced by wild birds which entered the folds in search of food.

Other epizootics

No report has been found in the literature of any other epizootic in terns or of avian influenza in other than domestic birds, i.e. chicken, turkey, duck (Pereira *et al.* 1965; Wells, 1963; Dinter, 1964), probably because they attract more attention and are more easily investigated.

Mass mortalities or epizootics affecting wild birds, including sea-birds other than

terns, have from time to time been reported but few of these were fully investigated and most of the reports are not very informative. In addition there appears to be too ready a tendency to ascribe these mortalities directly to adverse weather conditions.

Spread of Tern virus infection among terns

The rapid spread and high mortality rate seen in the epizootic in Common Terns were features in common with outbreaks of fowl plague in domestic poultry (Wells, 1963). The mode of spread of the infection could not be established but probably depended upon the close contact between birds at night at their roosting places in shallow water along estuaries, on sandbanks or on the small boats in harbours. The many birds affected during the epizootic had soiled vents and virus might have been spread via infected secretions or excretions. Tern virus was recovered from the nasopharynx and cloaca of experimentally infected chickens (Becker, 1964*b*). The role of ectoparasites is unknown. Ticks, mites and lice are known to harbour NDV for several days but are not known to transmit infection (Stubbs, 1959; Brandly, Moses, Jones & Jungherr, 1946).

SUMMARY

The aetiological agent of an epizootic among Common Terns (*Sterna hirundo*) in South Africa in 1961 was isolated from several sick birds and named Tern virus. It was classified on the basis of antigenic and morphological properties as a strain of avian influenza virus, Myxovirus influenzae A/Tern/South Africa/1961. The strain-specific antigen of Tern virus was unrelated to all known influenza strains with the single exception of Chicken/Scotland/1959 virus and the two viruses may be regarded as variants of the same strain. This relationship raised the interesting epidemiological possibility of the spread of infection between sea-birds and domestic poultry because the Common Tern migrates between Europe and South Africa.

I am indebted to Prof. A. Kipps for his counsel and unfailing support. My thanks are due to Misses E. Baker and S. van Olm for competent technical assistance; to the late Dr R. A. Alexander, Drs B. Jansen and K. Weiss of the Onderstepoort Veterinary Laboratories for their co-operation; to Dr J. Gear for the Influenza C virus; to Dr C. Chany, Dr M. S. Pereira and the United States Department of Agriculture for some of the immune sera; to University colleagues and R. Liversidge for the collection of sea-birds.

REFERENCES

Becker, W. B. (1963). The morphology of tern virus. *Virology* **20**, 318–27.

Becker, W. B. (1964*a*). Étude morphologique du virus Tern. Nucléocapsides énantiomorphes. *Annls Inst. Pasteur, Paris* **106**, 575–80.

Becker, W. B. (1964*b*). Tern virus. M.D. Thesis, University of Cape Town.

Blaxland, J. D. (1951). Newcastle disease in shags and cormorants and its significance as a factor in the spread of this disease among domestic poultry. *Vet. Rec.* **63**, 731–3.

Brandly, C. A., Moses, H. E., Jones, E. E. & Jungherr, E. L. (1946). Epizootiology of NDV in poultry. *Am. J. vet. Res.* **7**, 243–9.

DINTER, Z. (1964). Avian myxoviruses. In *Newcastle Disease Virus: An Evolving Pathogen*, pp. 299–312. Ed. R. P. Hanson. The University of Wisconsin Press.

DULBECCO, R. (1952). Production of plaques in monolayer tissue cultures by single particles of an animal virus. *Proc. natn. Acad. Sci., U.S.A.* **38**, 747–52.

FABIYI, A., LIEF, F. S. & HENLE, W. (1958). Antigenic analyses of influenza viruses by complement fixation. II. The production of antisera to strain-specific V antigens in guinea-pigs. *J. Immun.* **81**, 467–77.

HOYLE, L. (1952). Structure on the influenza virus. The relation between biological activity and chemical structure of virus fractions. *J. Hyg., Camb.*, **50**, 229–45.

JOENSEN, A. H. (1959). Disaster among fulmars (*Fulmaris glacialis*) and kittiwakes (*Rissa tridactyla*) in Danish waters in 1959. *Dansk Orn. Foren. Tidsskr.* **55**, 212–18.

LIEF, F. S. & HENLE, W. (1956*a*). Studies on the soluble antigen of influenza virus. I. The release of S antigen from elementary bodies by treatment with ether. *Virology* **2**, 753–71.

LIEF, F. S. & HENLE, W. (1956*b*). Studies on the soluble antigen of influenza virus. II. A comparison of the effects of sonic vibration and ether treatment of elementary bodies. *Virology* **2**, 772–81.

LIEF, F. S., FABIYI, A. A. & HENLE, W. (1958). Antigenic analysis of influenza viruses by complement fixation. I. The production of antibodies to the soluble antigen in guinea-pigs. *J. Immun.* **80**, 53–65.

MAYER, M. M., OSLER, A. G., BIER, O. G. & HEIDELBERGER, M. (1946). The activating effect of magnesium and other cations on the hemolytic function of complement. *J. exp. Med.* **84**, 535–48.

PEREIRA, H. G., TUMOVA, B. & LAW, V. G. (1965). Avian influenza A viruses. *Communicable Disease Centre Zoonoses Surveillance, Report Number* 5: *Animal Influenza*, pp. 26–31.

PORTERFIELD, J. S. (1960). A simple plaque-inhibition test for the study of arthropod-borne viruses. *Bull. Wld Hlth Org.* **22**, 373–80.

REED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty per cent end-points. *Am. J. Hyg.* **27**, 493–7.

RICHARDSON, G. M. (1941). Preservation of liquid complement. *Lancet* ii, 696–7.

ROWAN, M. K. (1962). Mass mortality amongst European Common Terns in South Africa in April–May 1961. *Br. Birds*, **55**, 103–14.

STUBBS, E. L. (1959). *Diseases of Poultry*, 4th ed., pp. 559–608. Ed. H. E. Biester and L. de Vries. Iowa State University Press.

WATERSON, A. P., HURRELL, J. M. W. & JENSEN, K. E. (1962). The fine structure of influenza A, B and C viruses. *Arch. ges. Virusforsch.* **12**, 487–95.

WELLS, R. J. H. (1963). An outbreak of fowl plague in turkeys. *Vet. Rec.* **75**, 783–6.

W.H.O. (1959). Respiratory Virus Diseases. First report of the Expert Committee. *Tech. Rep. Ser. Wld Hlth Org.* no. 170.

WILSON, J. E. (1950). *Vet. Rec.* **62**, 33. Quoted by Blaxland (1951).

J. Hyg., Camb. (1966), **64**, 321
Printed in Great Britain

Staphylococcal infection in thoracic surgery: experience in a subdivided ward

By O. M. LIDWELL, SHEILA POLAKOFF, M. PATRICIA JEVONS and M. T. PARKER

Cross-Infection Reference Laboratory, Central Public Health Laboratory, London, N.W. 9

R. A. SHOOTER, VALENTINE I. FRENCH and D. R. DUNKERLEY

Department of Bacteriology, St Bartholomew's Hospital, London, E.C. 1

(*Received* 26 *January* 1966)

INTRODUCTION

Most studies of the epidemiology of staphylococcal infection in British hospitals have been conducted in large open wards containing twenty or more patients. Such wards are now rarely built, as the present tendency is towards subdivision into separate rooms, each containing four patients or less. Among the reasons advanced to support this change is the impression that patients housed in small groups are less likely to suffer from sepsis due to cross-infection than those in large undivided wards.

The new surgical block in St Bartholomew's hospital consists of wards divided into a number of separate rooms. We now report on an investigation in the Department of Thoracic Surgery, which occupies one of these wards, to find out whether this subdivision was effective in preventing the spread of staphylococcal infection among the patients.

Architectural features

The ward under study occupied the third floor of the new block (see Fig. 1). It included a large room (A) containing ten to twelve beds, three four-bedded wards (B1, B2, B3) and four single-bedded cubicles (C1, C2, C3 and C4). One other four-bedded ward (BR) was used as a recovery room. Each room had a door leading to the corridor, but these doors were usually left open. All the rooms had windows which could be opened, and there was no artificial ventilation. The total volume of the ward was 65,000 cu.ft. The rooms occupied by patients had a total volume of about 34,000 cu.ft., or 1100 cu.ft. per patient when the ward was full. [1200 cu.ft./patient in A, 1000 cu.ft./patient in B1–3, and 1400 cu.ft./patient in C1–4.]

A set of twin operating theatres on the 5th floor served the whole block. They were shared by the Departments of Thoracic Surgery, Neurosurgery, and Ear, Nose and Throat Surgery. They had in common a 'clean' and a 'dirty' sterilizing room, which were separated from each other by a bank of double-ended autoclaves. The doors separating the theatres from the sterilizing rooms were kept shut

while not in use. The theatres were ventilated by positive-pressure at a rate of about twenty air changes an hour.

Ventilation of the ward

Ventilation and air movement within the ward were determined entirely by weather conditions and the opening of doors and windows, and varied considerably from time to time. The most consistent feature was a strong rising current of air

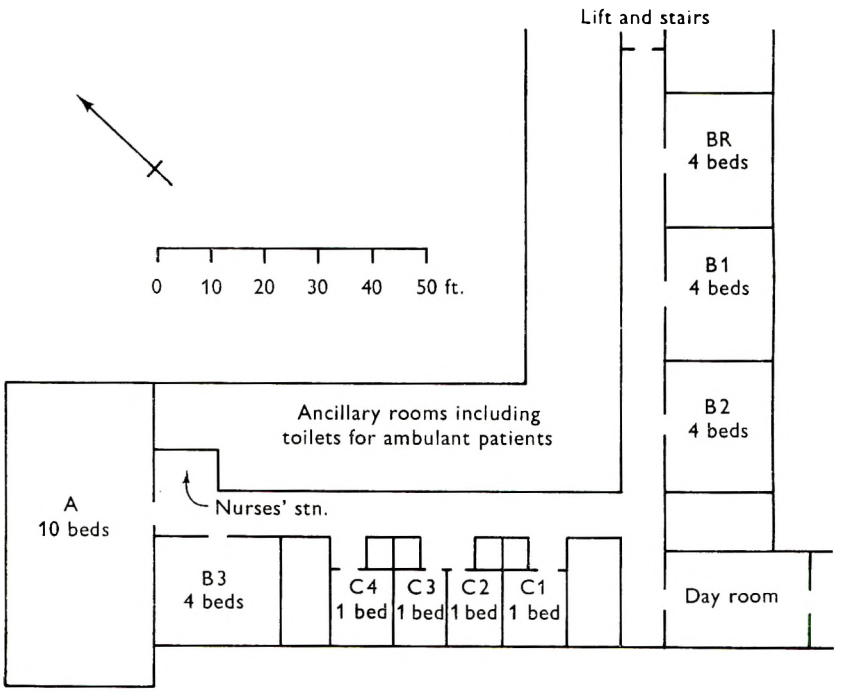


Fig. 1. Plan of the ward

up the stair-well, which entered the ward through the swing-doors at the north-east end of the corridor. The resulting pattern of air movement was explored by smoke tests on twenty-eight occasions spread over the whole of a calendar year. On no single occasion was the air flow between the corridor and the rooms of the ward uniformly in one direction, i.e. from the corridor to all the rooms or vice versa, and on only four occasions was it in the same direction for even as many as two-thirds of the rooms. In all, 33 % of the 252 observations on individual rooms showed air flow from the room into the corridor, 27 % flow in the reverse direction, and the remaining 40 % convectional exchange between room and corridor. The volumes of air involved in these movements were not measured, but a velocity as low as 20 ft. per min. across the upper or lower half of a door opening—and the room doors were open for the greater part of the day—will move as much as 6000 cu.ft. of air per hour.

More detailed quantitative studies were made on three occasions using nitrous oxide as a tracer gas. These confirmed that substantial volumes of air were moving

between the rooms and the corridor. The detailed patterns of air movement were complex. In general, however, the figures indicated an average level of exposure to airborne contamination of the order of one-third of that obtained in a similar experiment carried out in an open ward. This reduction represents the combined effect of restricted mixing in the subdivided ward and of differences in effective ventilation between the two wards. The ventilation of a complex system can be represented only approximately by a single figure, but for this ward on three occasions the average value appeared to be about $4\frac{1}{2}$ air changes per hour, reckoned on the volume of the rooms containing patients and the corridor.

METHODS

Patients

The ward contained both male and female patients. The large room (A) was nearly always occupied by male patients. The allocation of the four-bedded wards (B 1–3) and the single-bedded cubicles (C 1–4) to male and female patients was made according to the number of patients of either sex and their surgical requirements. After operation, most of the patients spent some time in the recovery room (BR). They usually stayed there for less than 24 hr., but a few requiring intensive therapy were there for several days.

Wound dressings. Dressings were removed on the third post-operative day. Wounds showing no clinical signs of infection were sprayed with Nobecutane (Evans Medical Supplies) and left uncovered. Stitches were removed on the eighth day, and Nobecutane was again applied.

Determination of sepsis. A wound was considered to be septic only when visible pus was present.

Treatment with antibiotics. Nearly all patients subjected to thoracotomy received injections of benzyl penicillin (1 million units 8-hourly) for 4 days, followed by procaine penicillin (600,000 units 12-hourly) for 3 days. Patients for whom artificial circulation was used were given benzyl penicillin (1 million units 6-hourly) and streptomycin (0·5 g. 12-hourly) by injection for 7 days; some of them were also given penicillin V orally for a further month.

In all, 506 of 714 patients (71 %) and 490 of 618 patients who underwent a surgical operation (80%) received an antibiotic while in hospital. Patients who were operated upon and received no antibiotic were in general subjected to minor procedures, or to ones in which there was no cutting of the tissues. Indeed, only six patients who had a major cutting operation did not receive an antibiotic at some time. The percentages of patients receiving individual antibiotics were as follows: penicillin 63, streptomycin 14, tetracycline 7, chloramphenicol 4, methicillin or cloxacillin 3, ampicillin 2, any other antibiotic less than 1.

Staff

The staff consisted of two consultant surgeons, one or two registrars and two house-surgeons. In addition to the sister in charge, the day staff included six staff-nurses and nine nurses in training. At night there were one staff-nurse and six

student nurses in the ward. About fifteen other professional and technical persons (anaesthetists, respiratory physiologists, physiotherapists, etc.) worked intermittently in the ward.

Bacteriological examinations

Nasal swabs were taken from patients on admission and thereafter weekly on a fixed day. A further swab was taken on the day of each operation. Wounds were swabbed at the first dressing, and again when the stitches were removed. Further swabs were taken if necessary. If there was clinical evidence of sepsis elsewhere, the appropriate specimens were sent to the laboratory. Coagulase-positive staphylococci (*Staphylococcus aureus*) were tested for sensitivity to antibiotics and phage-typed. Nasal swabs were also taken weekly from all members of the surgical, nursing, and other staff of the ward.

Air-sampling

Air-sampling was carried out on 28 days, at approximately fortnightly intervals. On each day, three slit-samplers were run simultaneously for two successive periods of 1 hr., in the course of each of which 108 cu. ft. (3·06 cu.m.) of air were sampled by each machine on a phenolphthalein-phosphate serum-agar plate. The first sampler was always in the 10-bedded ward (A). The second was placed for the first hour alternately in one of the 4-bedded rooms (B 1 or B 2), and for the second hour was moved into the other. The third was placed for the first hour alternately in C 1 or C 2 and was moved for the second hour from C 1 to C 3 or from C 2 to C 4.

Colonies resembling *Staph. aureus* were subcultured and tested for coagulase production. Up to ten coagulase-positive cultures from each plate were phage-typed and tested for resistance to penicillin and tetracycline. When more than ten colonies of *Staph. aureus* were present on a plate, strains were assumed to be distributed in the sample in the same ratio as in the ten colonies that had been phage-typed.

Method of analysis

We tried to determine the source of the *Staph. aureus* strains which caused septic infection or colonization, and to relate the risk of either event to the position in the ward of both donor and recipient, and to the presence of the infecting organism in the air. Strains of *Staph. aureus* isolated from lesions, from nasal swabs, and from the air were therefore matched, both as regards their phage-typing patterns and their sensitivity to penicillin and tetracycline. In determining the source of a staphylococcus which was acquired by a patient, or was isolated from the air, we considered only those persons present in the ward at the time who were judged to be carrying an indistinguishable organism.

A major difficulty was that often two or more patients were carrying indistinguishable strains of *Staph. aureus* at the relevant time. In an earlier investigation (Shooter *et al.* 1963) we accepted that, when there were two or more alternative sources for an organism, the nearest should be accepted as the true source. This introduced an element of bias into our results. This time we based our analysis on unambiguously located sources, that is to say, organisms acquired by a patient,

or organisms found in the air, which were carried by one or more members of the staff, or by a single patient-source (see (*f*) below). An exception we made was that an organism carried by a patient at one body-site was always considered to be the source if he or she subsequently acquired it elsewhere.

Because we cultured the nose and lesion of patients, and the nose of members of the staff, only at intervals, we had to adopt certain conventions, rather like those used by Williams *et al.* (1962), about the carriage-state and location in the ward of the patients on each intervening day of the investigation. They are summarized as follows.

(*a*) When two weekly nasal swabs gave different results, a change in carriage-state was deemed to have taken place on the day following the mid-point of the intervening period; but if the number of days between two swabs was odd, the day after the middle day was the one on which the change was considered to have occurred. A few patients were not swabbed on admission, but had a positive first swab. They were considered positive for 3 days before this.

(*b*) The interval between successive nasal swabs of patients occasionally exceeded 7 days. If an organism was present in one swab and absent from the other, its carriage was assumed for half the intervening period, or for a total of 7 days, whichever was the shorter. A few patients were omitted on the swabbing day just before their discharge from hospital. When the last swab to be taken was positive, carriage was assumed up to the day of discharge only if there had been two previous positives for the same strain.

(*c*) Staff swabbing was necessarily more intermittent, because of temporary transfers to other wards and periods of leave. Carriage was therefore assumed for not more than 13 days on either side of a positive swab, and a change in carrier-state was taken to have occurred midway between two relevant swabs.

(*d*) A swab or other specimen from a lesion was taken as a central point, with carriage of 3 days on either side, unless it was preceded or followed by another swab positive for the same organism, when carriage was assumed for up to 14 days between positive swabs.

(*e*) When determining the source of an acquisition, the earliest date of appearance of the organism at any site was the one considered.

(*f*) A single patient-source was usually the only patient carrying the particular strain at the relevant time. On two occasions, however, when two patients in the same subdivision of the ward were carrying indistinguishable strains they were regarded as a single source.

(*g*) A source for a particular infection was considered to be unambiguously located only when neither donor nor recipient had changed their position in the ward since the last swabbing day. Thus a change in bed position caused ambiguity for the six days lying between successive swabbing days.

RESULTS

Incidence of sepsis and wound-colonization

We observed 714 patients in the 20 months between October 1962 and July 1964, and 618 of them underwent 690 surgical operations. In calculating the incidence of post-operative sepsis we excluded 190 operations in which the tissues were not cut, and a further 38 where the patient died within 3 days of operation or where for some reason no wound swab was taken. Wound sepsis followed 11 of the remaining 462 cutting operations, and *Staph. aureus* was isolated from 6 of the swabs, giving a wound-sepsis rate of 2·4 and a staphylococcal wound-sepsis rate of 1·3 per 100 operations. There was one death directly attributable to staphylococcal wound sepsis. *Staph. aureus* was isolated from a further 15 wounds in which there was no clinical evidence of sepsis. Thus, the total wound-colonization rate was 21 in 462 operations, or 4·5 per 100 operations (Table 1).

Table 1. *Staphyloccocal wound sepsis and wound colonization, excluding tracheostomy wounds*

(In parenthesis: rate per 100 operations. Cardiac (*a*): operations with artificial circulation and profound hypothermia. Cardiac (*b*): without hypothermia.)

	Number performed		Staphylococcal wound sepsis		Total staphylococcal colonization*	
	M	F	M	F	M	F
Operations						
Cardiac (*a*)	53	32	2 (3·8)	0	6 (11·3)	3 (9·4)
Cardiac (*b*)	42	132	1 (2·4)	0	1 (2·4)	2 (1·5)
Pulmonary	103	33	2 (1·9)	0	6 (5·8)	0
Other cutting operations	36	31	1 (2·8)	0	3 (8·3)	0
Total cutting operations	234	228	6 (2·6)	0	16 (6·8)	5 (2·2)
	462		6 (1·3)		21 (4·5)	

* Staphylococcal wound sepsis plus colonization of wounds.

This table shows also that staphylococcal wound sepsis was found only among male patients, in whom the incidence was 2·6 per 100 operations. Total colonization of wounds with *Staph. aureus* was also more frequent in male than in female patients (respectively 6·8 and 2·2 per 100 operations).

Tracheostomy wounds were not included in these totals, because the clinical significance of the presence of *Staph. aureus* in them was difficult to assess. The organisms present were usually those also isolated from the sputum.

Staphylococcal sepsis occurred at sites other than the operation wound in 20 of the 714 patients (2·8%), and was more common in males (17 out of 393: 4·3%) than in females (3 out of 321: 0·9%). It was seen only in those who had had an operation. In all, six of these patients were seriously ill, and four suffered from empyema, but no death was directly attributable to any of these infections.

Post-operative wound sepsis was, therefore, not common in the Thoracic

Surgery unit, despite the severity of many of the operations performed. Indeed, sepsis rates were lower than those seen in general surgical wards in the same hospital (Williams *et al.* 1962; Shooter *et al.* 1963), which were of the order of 6 % for total wound sepsis, 4–5 % for staphylococcal wound sepsis, and 4 % for other staphylococcal sepsis.

Staphylococcus aureus *in the ward air*

We examined the staphylococci isolated from the ward air for evidence that the design of the ward prevented or hindered the dispersal of staphylococci by the airborne route. The average number of staphyloccoci in the air on the 28 days of sampling was only 35 *Staph. aureus* per 1000 cu.ft. (28·3 m.^{3}), several times lower than the values we had observed earlier in open surgical wards in St Bartholomew's Hospital (Noble, 1962). Numerous measurements in three wards in the years 1956–61 gave average counts of 220, 180 and 180 per 1000 cu.ft. respectively. In 1961, one of these wards was divided by a partition (Shooter *et al.* 1963), and the following year the counts were rather lower, with means of 70 and 80 per 1000 cu.ft. on the two sides of the ward.

There were considerable variations in the count from day to day, and from one room to another in the thoracic surgery ward, but there were few 'broadcasts' of staphylococci resulting in very high counts. Indeed 90 % of all counts were less than 80/1000 cu.ft. The corresponding 90-percentile figure for the open wards (Noble, 1962) was 600/1000 cu.ft. One really high count (1150/1000 cu.ft.)—twice as great as the next highest—was obtained in a cubicle occupied by a moribund patient.

Table 2. *Recovery of airborne* Staphylococcus aureus *in relation to presumed source*

(Colonies per 1000 cu. ft. air attributable to a single source-carrier. (No air-samples were collected in rooms B3 and BR.))

Recovery from air. Room	Location of source carrier. Room: A	B1, B2	B3, BR	C1, C2, C3, C4	Staff	No known source*
A	**5·25**	0·23	0·23	1·68	0·24	1·74
B B1, B2	0·80	0·68	0·11	1·68	0·07	3·29
B Same room	—	**4·73**	—	—	—	—
C C1, C2, C3, C4	0·62	2·59	< 0·11	3·36	0·18	2·82
C Same room	—	—	—	**75·2** (**21·9**†)	—	—

* Colonies per single strain, source carriers being unidentifiable

† Excluding one exceptionally high count.

Figures in bold are recoveries in the same room as the presumed source.

Dissemination of staphylococci from known sources

Staphylococci isolated from the air were allotted when possible to known carriers in the ward. A total of 630 isolations had been made; 410 of them (65 %) could be attributed to a single carrier-source, 69 (11 %) appeared to have no source in the ward, and 151 (24 %) were strains for which there was more than one carrier-source.

Our main analysis was confined to those for which there appeared to be a single source.

Table 2 shows the recovery of airborne organisms in the same room as the source, and in other rooms, expressed as colonies recovered from 1000 cu.ft. of air. A single carrier-source produced a much higher count in the room in which he or she was placed than in other rooms. The difference was of the order of tenfold, whether the patient's room was a large one or a small one. Dissemination by members of the staff was detected at a rather low level throughout the ward.

Table 3. *Recovery of airborne* Staphylococcus aureus *in relation to antibiotic resistance*

(Colonies per 1000 cu. ft. air attributable to a single source carrier. S = sensitive to penicillin and tetracycline. P = resistant only to penicillin. T = resistant to penicillin and tetracycline; or to tetracycline alone.)

Location of source carrier	Antibiotic resistance of strains isolated			All organisms
	S	P	T	
Patients: same room	18·2 (4·1*)	4·4	33·3	15·2 (7·5*)
Patients: other rooms	0·75	0·4	2·42	0·84
Staff	0·04	0·23	< 0·40	0·16
No known source	2·50	2·32	7·20	2·62

* Excluding one exceptionally high count.

Table 4. *Average number of nasal carriers of* Staphylococcus aureus *to whom a patient was exposed (a) in the same room, (b) in other rooms, and (c) among the staff*

Exposure to	Antibiotic resistance of carried strain			All carriers	All persons
	S	P	T		
Patients: same room	0·57	0·52	0·32	1·42	4·6
Patients: other rooms	2·92	2·78	1·66	7·35	24·4
Staff	2·54	9·71	1·14	13·39	34·4

The last column of Table 2 shows that strains for which no source could be identified appeared in larger numbers in sections B and C of the ward than in section A. This supports the view that a proportion of them were organisms reaching the ward in the air from the stair-well.

When we examined the antibiotic resistance of the airborne organisms emanating from known sources (Table 3) we found that the dispersal of multiple-resistant *Staph. aureus* by carriers among the patients was several times as profuse as the dispersal of sensitive organisms or of those resistant only to penicillin.

Although a patient in a room was more heavily exposed to a source of airborne infection in that room than to one in another room, the number of potential sources in the other rooms exceeded those in the same room (Table 4). We therefore attempted to compare the total amount of exposure of the average patient to

sources in the same and in different rooms. The relative contributions of patients in the same and in other rooms varied in the different rooms according to the number of patients they contained. Since, however, the number of cases of sepsis and of nasal acquisition observed in the investigation was not very large, it was necessary to combine them into a few groups in order to obtain reasonable numbers in each. From the average carrier-rate of patients in each room and the figures for airborne dispersal given in Table 2 we calculated the effective exposure to airborne organisms derived from all patients in the same and in different rooms, from the staff, and from unknown sources, further subdividing this according to the antibiotic resistance of the organisms (Table 5). Total exposure to potential patient-sources in other rooms (6·3/1000 cu.ft.) was about two-thirds of the total exposure to sources within the room (9·8/1000 cu.ft.). For resistant strains, however, sources in other rooms contributed only about one-half as much as sources within the same room, while for sensitive strains they contributed twice as much. This would imply either that sensitive strains were disseminated more readily from room to room or, more probably, that the carriers of resistant strains were not randomly distributed through the rooms of the ward but tended to be grouped in the same room.

Table 5. *Effective exposure to airborne staphylococci. Colonies of* Staphylococcus aureus *per* 1000 *cu.ft. air*

	Antibiotic resistance of strain			
Source of organisms	S	P	T	All organisms
Patients: same room	1·1	1·8	6·9	9·8
Patients: other rooms	2·0	1·1	3·2	6·3
Staff	0·1	0·9	< 0·1	1·0
No known source	3·9	4·0	0·8	8·7
Total	7·1	7·8	10·9	25·8

Nasal carriage of Staphylococcus aureus

Unexpected changes were found in the nasal carriage of *Staph. aureus* by patients during successive weeks of their stay in hospital (Fig. 2). In the first 2 weeks there was a reduction in the total carriage-rate from 38 % to 25 %. This was accounted for almost entirely by a loss of sensitive organisms. This loss continued, though at a rather slower rate, for the rest of the stay of patients in the ward, so that between three-quarters and four-fifths of all sensitive organisms had disappeared by the end of the 6th week. A substantial number of patients carried tetracycline-resistant organisms on admission, but the rise from 6 % to a maximum of 9 % while in hospital was a relatively small one.

These findings contrasted sharply with our earlier experiences in open surgical wards (Williams *et al.* 1962) in which the total carrier-rate increased with the duration of stay in hospital, and the percentage of patients carrying staphylococci resistant both to penicillin and to tetracycline increased at least sevenfold in 6 weeks (Fig. 3C–E). The findings in the current investigation (Fig. 3A) exhibit in rather more extreme form the situation we observed among patients nursed in cubicles communicating with the open air, many of whom were also receiving

antibiotics (Parker, John, Emond & Machacek, 1965; see also Fig. 3B). Here, the total carrier rate was substantially unchanged, many sensitive staphylococci were lost, and the increase in multiple-resistant organisms was trivial.

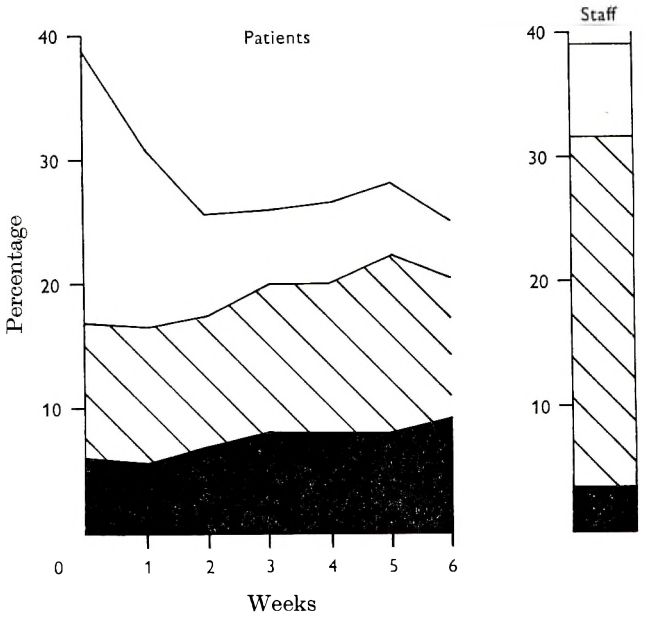


Fig. 2. Nasal carriage of *Staph. aureus* by patients during each week of stay in the thoracic surgery ward. Average rate of nasal carriage by members of the staff. □, Sensitive to penicillin and tetracycline; ▨, resistant to penicillin only; ■, resistant to tetracycline

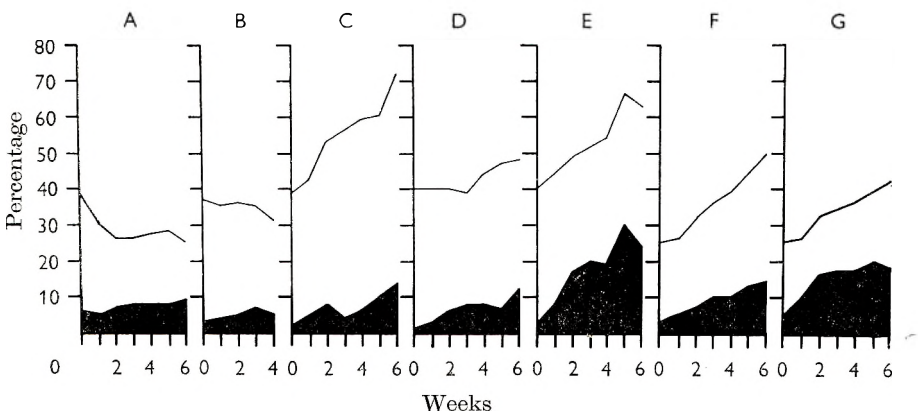


Fig. 3. Nasal carriage of *Staph. aureus* by patients in various hospital wards. Percentage positive in each week of stay in the ward. Upper line: all strains of *Staph. aureus*. Lower line: tetracycline-resistant *Staph. aureus*. [A, B, F, and G: all tetracycline-resistant strains; C, D, and E: all strains resistant to penicillin and tetracycline.] A, This investigation; B, patients in cubicles (Parker *et al.* 1965); C, D, E, three open surgical wards (Williams *et al.* 1962). F, medical wards: patients not receiving antibiotics; G, Medical wards: patients receiving antibiotics. (F, G. P.H.L.S. Cross-Infection Committee (unpublished results).)

It appeared, therefore, that a large proportion of the sensitive staphylococci present on admission to the thoracic surgery ward were being removed or suppressed by antibiotic treatment, and were not being replaced by multiple-resistant organisms, as so frequently happens in other wards. This was remarkable in view of the very high rate of administration of antibiotics, and the often-repeated observation that multiple-resistant staphylococci are more rapidly acquired by those who receive antibiotics than by those who do not (Fig. 3F, G.)

Apparent acquisition of new strains of Staphylococcus aureus

Ninety-six strains of *Staph. aureus* not found on admission were subsequently isolated from the nose swabs of patients. The rate of apparent acquisition in 2790 patient-weeks of exposure was therefore 3·44 per 100 patient-weeks, a figure little more than half of that seen in patients isolated in cubicles (Parker *et al.* 1965). There is, however, reason to believe that a considerable proportion of 'apparent' acquisition is an artifact due to the intermittent isolation of organisms present before admission; most of this spurious acquisition is of sensitive organisms, or organisms resistant only to penicillin, and is first detected in the swabbings immediately following the admission swab. A high rate of antibiotic administration would tend to reduce this spurious element in acquisition, and lower the total rate of apparent acquisition. The rate of apparent acquisition of multiple-resistant staphylococci, which are less often found on admission, is therefore a better index of the exposure to infection in hospital. In the present investigation, this figure was 1·33 per 100 patient-weeks, which corresponded closely with the figure of 1·40/100 patient-weeks for patients in cubicles. Comparable figures for the apparent acquisition of multiple-resistant organisms in open wards range from 2 to 12 per 100 patient-weeks (Parker *et al.* 1965).

Sources of nasal acquisition

Table 6 summarizes our attempts to find sources for the 96 apparent acquisitions of *Staph. aureus*. One or more possible sources were identified for 49 of them (51 %), but there were a number of uncertainties. Three acquisitions could be attributed unambiguously to patients in the same room, and 12 to patients in other rooms, but nine further acquisitions were from patients of uncertain location, either because donor or recipient had moved recently, or because two or more patients were carrying identical organisms. Nine acquisitions could have been either from patients or from members of the staff and 16 were definitely attributable to the staff. We attempted to clarify this by distributing the acquisitions where the source or its location were uncertain according to the observed distribution of acquisition from sources with unambiguous locations. The picture that emerges is that 3–4 times as many new strains were acquired from patients in other rooms—and from members of the staff—as from patients in the same room.

There was a preponderance of antibiotic-sensitive strains among the 47 for which there was no known source. This, and the fact that most of the apparent acquisitions of sensitive organisms occurred early in the stay in hospital, is in conformity with the view that many of them were spurious. To estimate what

proportion of those with no known source were 'true' acquisitions we divided them into two parts; (*a*) a fraction with a distribution of antibiotic sensitivity similar to that for acquisitions from known sources, and (*b*) a fraction with a distribution of antibiotic sensitivities similar to that in the patients' admission swabs (Table 6, lines 7 and 8). These fractions might be expected to correspond respectively to 'true' acquisitions from undetected sources in the hospital, and to spurious acquisitions. The result suggested that 24 of the 47 'acquisitions' with no known source—including 14 of the 16 penicillin-sensitive organisms—were spurious.

Table 6. *Source of nasal acquisitions of* Staphylococcus aureus

((1) The figures in parentheses were obtained by distributing those acquisitions where the source or its location were uncertain (lines 3 and 4) according to the observed distribution of acquisition from sources whose location was unambiguous. (2) Acquisition from no known source has been divided into two fractions with distribution of antibiotic sensitivities similar to (*a*) that for acquisition from known sources and (*b*) that found in admission-swabs (see text).)

Source	Antibiotic resistance of acquired strain			Total acquired
	S	P	T	
1. Patients in same room	0 (0·6)	0 (0)	3 (5·2)	3 (5·8)
2. Patients in other rooms	0 (0·6)	4 (6·5)	8 (13·8)	12 (20·9)
3. Patients, location uncertain	1	0	8	9
4. Patients or staff	1	8	0	9
5. Staff	3 (3·7)	9 (14·5)	4 (4·0)	16 (22·2)
6. No known source	16	17	14	47
7. No known source (*a*)	2	10	11	23
8. No known source (*b*)	14	7	3	24
Total	21	38	37	96

Table 7. *Rates of acquisition of* Staphylococcus aureus *from single sources*

(Rate per 1000 patient-weeks exposure per source. The figures in parentheses are derived from an analysis confined to the 31 acquisitions where source and recipient were unambiguously situated. The remaining figures were obtained after redistribution of the acquisitions from known sources where the location was ambiguous (see Table 6). Figures in square brackets are based on insignificant numbers of acquisitions (< 1).)

Source	Antibiotic resistance of acquired strains			All acquisitions
	S	P	T	
1. Patients in same room	[0·35]	[0·38]	5·9	1·48 (1·93)
2. Patients in other rooms	[0·07]	0·84	3·1	1·04 (0·97)
3. Staff	0·47	0·54	1·2	0·58 (0·70)
4. All known sources	0·27 (0·32)	0·58 (0·70)	2·69 (2·54)	0·79 (0·82)

Rates of acquisition from single carriers

Table 7 shows the nasal acquisition rates per 1000 patient-weeks' exposure per known source. The figures in the body of the table were derived from all the acquisitions for which there was a source, after redistribution of those that were ambiguously located. Those in parentheses were obtained from an analysis

confined to acquisitions where source and recipient were unambiguously located throughout the relevant period. The numbers are small, but serve to confirm the results of the less complicated analysis. They suggest that acquisition from a single carrier occurs about twice as often when he is in the same room as when he is in another room. This is about the same as the difference previously observed between adjacent and remote beds in an open ward (Williams *et al* 1962). Acquisitions from a carrier on the staff occurred at about half the rate of acquisitions from carriers in other rooms. This may reflect the fact that members of the staff spend only part of the day in the ward.

There was a numerical preponderance of tetracycline-resistant staphylococci among those acquired from other patients, and of organisms resistant only to penicillin among those acquired from the staff (Table 6). The rate of acquisition (Table 7) of tetracycline-resistant organisms per patient-source was fairly high (5·9/1000 patient weeks in the same room), but the rate for penicillin-resistant organisms per staff-source (0·54/1000 patient-weeks) was less than the average for all acquisitions from single sources. This suggests that patients with tetracycline-resistant organisms were relatively infectious, and that carriers of penicillin-resistant organisms on the staff, although numerous, were not individually very dangerous.

Exposure to airborne staphylococci and risk of nasal acquisition

The distribution of sources of nasal acquisition (Table 6) is closer to the distribution of the number of carriers (Table 4) than to the amount of exposure to airborne organisms derived from them (Table 5). This is compatible with a non-linear relationship between dose and risk of infection such as would result from differences between patients in their susceptibility to colonization or between staphylococcal strains in their transmissibility. Variations in the dose of organisms would then produce proportionately smaller changes in the risk of infection (Lidwell, 1963). Figure 4 shows the relationship between the risk of nasal acquisition and the exposure to airborne organisms for all strains and for tetracycline-resistant strains. The results are consistent with the risk varying as the one-fifth power of the exposure. For this population, an average inhaled dose of 10 airborne staphylococcal particles of a single strain corresponded to a probable risk of nasal acquisition of about 1 in 800 or 1 in 300 for tetracycline-resistant staphylococci.

Sources of staphylococci causing sepsis and wound colonization

Forty-two strains of *Staph. aureus* were isolated from wounds or from staphylococcal lesions elsewhere. Table 8 shows their presumed sources. Twenty of them (48 %) were isolated from the patient on admission to hospital and must therefore be considered self-infections; nine of them, however, were due to *Staph. aureus* strains that were resistant both to penicillin and to tetracycline, and can probably be attributed to a previous hospital admission. Eight (19 %) were from unknown sources. This number is high, but included a sudden 'burst' of four 83A infections which could not be traced, and were possibly due to failure to detect a person carrying the organism at a site other than the nose.

The remaining 14 infections were undoubtedly acquired from sources in the ward. The six that were probably acquired from other patients were, however, too few to allow any conclusions to be drawn about the relative importance of sources of infection in the same or in different rooms. This illustrates the difficulty of investigating clinical sepsis when its incidence is low.

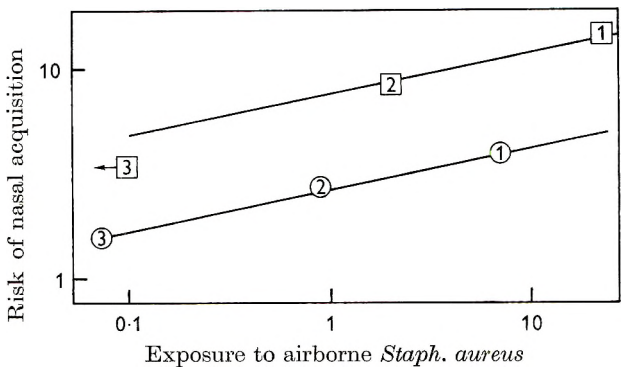


Fig. 4. Relation between the risk of nasal acquisition and the exposure to airborne *Staph. aureus*. Logarithmic scales for both co-ordinates. Risk of nasal acquisition: acquisitions per potential source (carrier) per 2790 patient weeks. Exposure to airborne *Staph. aureus*: colony count per carrier per 1000 cubic foot of air sampled. Lines drawn with a slope of 0·20. ○, All strains; □, tetracycline-resistant strains. Location of carriers: 1, patients in the same room; 2, patients in other rooms; 3, staff.

Table 8. *Presumed source and antibiotic resistance of* Staphylococcus aureus *strains which caused wound sepsis, wound colonization and other septic lesions*

	Presumed source					
	Self	Another patient or patients	Staff	Staff or patient	Not known	Total
Infection						
Wound sepsis	3	1	1	0	1	6
Wound colonization	7	2	1	3	2	15
Other septic lesions*	10	3	3	0	5	21
Sensitivity of infecting organism						
S	2	0	0	0	0	2
P	9	0	2	3	1	15
T	9	6	3	0	7	25
Total	20	6	5	3	8	42

* Two organisms from one lesion.

DISCUSSION

The incidence of staphylococcal sepsis was low in this ward, but it proved unexpectedly difficult to decide why this was so. There are great differences in the reported incidence of post-operative sepsis following operations on the chest, but

there seems no reason to suppose that the risk of infection in this branch of surgery is inherently small. Thus, while Hansen & Eriksen (1964) recorded a total sepsis-rate of less than 2% following thoracic operations over a 10-year period, others have reported the incidence of staphylococcal sepsis to be as high as 8–11% (Blowers, Mason, Wallace & Walton, 1955; Report, 1960; Laurell & Lindbom, 1961; Bassett *et al.* 1963; Lindbom, 1964). Many factors, including selection of patients, operative techniques, the use of antibiotics, ward procedures and the methods of recording sepsis may have contributed to these differences. In addition, high rates of sepsis have sometimes been associated with particular sources of infection in the operating theatre (Blowers *et al.* 1955; Bassett *et al.* 1963).

We found that staphylococcal sepsis and wound colonization were both more frequent in male than in female patients. The males were usually nursed in the 10-bedded ward and the females in smaller units, but the males were on the whole older than the females, underwent different operations, and received more antibiotics. We are unable, therefore, to draw any conclusions from this observation.

Not only was the total incidence of sepsis low in our investigation, but nasal colonization, including the acquisition of endemic strains of hospital staphylococci, was infrequent, and the numbers of staphylococci present in the air of the ward were much lower than those we have previously observed in other wards in the hospital.

While the natural ventilation rate of the ward was probably greater than usual and the relatively spacious construction resulted in a low patient density, these differences were not sufficiently great to provide in themselves a convincing explanation of the low air counts.

The administration of antibiotics will usually reduce the level of carriage of sensitive strains even when it does not eliminate them from the nose of the patients concerned. When this occurs the amount of dispersal of these strains into the environment is also reduced (Bøe & Solberg, 1965). Usually where there has been extensive prophylactic use of antibiotics the removal or suppression of the sensitive strains has been accompanied by a widespread acquisition of resistant 'hospital' strains. In this ward we saw a massive removal of the sensitive staphylococcal population following antibiotic treatment but very little replacement by resistant strains, although potential sources of infection with these were present in the ward for most of the time. Since many patients had been in hospital previously a substantial percentage were admitted carrying such strains.

The low air counts would seem then to be mainly due to the suppression, by antibiotics, of widespread dispersion by carriers of sensitive strains unaccompanied by the appearance of carriers of resistant strains. The rate of dispersal of their staphylococci by carriers of resistant strains was, on the average, about three times as great as that of carriers of sensitive strains (Table 3).

In looking for reasons to explain the low rate of spread of staphylococci within this ward the subdivided construction needs to be considered first. Subdivision could reduce the spread of micro-organisms in two ways.

It might influence nursing procedures in such a way as to reduce the risk of

contact-transfer from patients in one room to patients in another, e.g. by increasing the probability of the nurses washing or performing some other duty between attending to patients in different rooms. There was however more acquisition of staphylococci from patient-carriers situated in other rooms than from those in the same room. It does not, therefore, seem likely that changes of this kind in nursing procedure, if they occurred, were such as to influence the spread of staphylococci.

Alternatively, the subdivision might isolate the patients from each other by hindering the movement of airborne organisms from one part of the ward to another. Smoke and tracer gas studies, however, together with air sampling for staphylococci, showed that in fact there was still substantial interchange of air between the several parts of the ward. Although in some wind and weather conditions the upward current of air in the stair-well of the building, which entered the ward passage through the swing doors at the end of the corridor, swept through this and passed out through the several rooms in a regular manner, there was usually substantial exchange of air between these rooms and the corridor, caused by thermal differences. As in a previous investigation where a ward was divided into two parts by a partition with sliding doors (Shooter *et al.* 1963), it became clear that division of a ward unaccompanied by ventilation to control the direction of air movement does little to prevent the spread of staphylococci from room to room by the aerial route. In fact patients in one room were exposed to organisms from sources in other rooms almost as much as if these had been sources at the far end of a large open ward. We are left therefore without any simple explanation of the undoubted good results obtained. The best that we can offer is the suggestion that the combination of subdivision, spaciousness and above average ventilation reduced the risk of spread below a threshold level so that the generous administration of antibiotics was not accompanied by widespread colonization with resistant strains.

The widespread prophylactic use of antibiotics is undoubtedly hazardous, although it may be justified in a few situations, of which major thoracic surgery is widely held to be one (Eriksen & Hansen, 1964). If it is practised the patients should be segregated from each other as strictly as possible and no antibiotic should be given to a patient already colonized with an organism resistant to it. This second condition was not always observed in our studies, which may have contributed to the fact that our carriers of multiple-resistant strains disseminated more heavily than did the carriers of sensitive organisms; many were undoubtedly receiving antibiotics to which their nasal strains were resistant. This would have been likely to have increased profuseness of carriage and the extent of dispersion (Ehrenkranz, 1964).

SUMMARY

We studied the incidence of staphylococcal infection in a thoracic surgery ward which consisted of a number of separate rooms, and inquired whether the subdivision of the ward was responsible for the unusually low sepsis-rate.

The airborne dissemination of *Staphylococcus aureus* from one room to another appeared to be little less than that in an open ward; but the total number of *Staph. aureus* in the air was very low.

Most of the patients received prophylactic antibiotics. The nasal carrier-rate of *Staph. aureus* by patients fell greatly during their stay in the ward. There was a progressive disappearance of sensitive organisms and little acquisition of multiple-resistant organisms.

When there are urgent clinical grounds for the lavish use of antibiotics, the dangers appear to be reduced by effective segregation of the patients from each other.

We are grateful to our surgical colleagues, Mr O. S. Tubbs and Mr I. M. Hill, for permission to study their patients, and to the Treasurer and Governors of St Bartholomew's Hospital for financial support. We wish also to thank the Sister and nursing staff of the ward for their co-operation, and Miss Anne Brooke, Miss Madeleine John, B.Sc., Mrs Janet Robertson, B.Sc., and Miss Veronica Williams, B.Sc., for assistance in our laboratories.

REFERENCES

BASSETT, H. F. M., FERGUSON, W. G., HOFFMAN, E., WALTON, M., BLOWERS, R. & CONN, C. A. (1963). Sources of staphylococcal infection in surgical wound sepsis. *J. Hyg., Camb.*, **61**, 83.

BLOWERS, R., MASON, G. A., WALLACE, K. R. & WALTON, M. (1955). Control of wound infection in a thoracic surgery unit. *Lancet* ii, 786.

BØE, A. J. & SOLBERG, C. A. (1965). Behandling av nasale stafylokokkbaerere med fucidin. *Tidsskr. norske Laegeforen.* **85**, 527.

EHRENKRANZ, N. J. (1964). Person-to-person transmission of *Staphylococcus aureus*; quantitative characterization of nasal carriers spreading infection. *New Engl. J. Med.* **271**, 225.

ERIKSEN, K. R. & HANSEN, J. L. (1964). Prophylactic use of antibiotics in surgery of the lung. *Acta chir. scand.* **128**, 651.

HANSEN, J. L. & ERIKSEN, K. R. (1964). Postoperative infection in thoracic surgery. *Acta chir. scand.* **128**, 698.

LAURELL, G. & LINDBOM, G. (1961). Wound infections due to *Staphylococcus pyogenes* in a thoracic surgical unit. *Acta chir. scand.* **121**, 165.

LIDWELL, O. M. (1963). Methods of investigation and analysis of results. In *Infection in Hospitals*, p. 43. Ed. R. E. O. Williams and R. A. Shooter, Oxford: Blackwell Scientific Publications.

LINDBOM, G. (1964). Studies of the epidemiology of staphylococcal infections. II. Staphylococcal infections in a thoracic surgery unit. *Acta chir. scand.* **128**, 421.

NOBLE, W. C. (1962). The dispersal of staphylococci in hospital wards. *J. clin. Path.* **15**, 552.

PARKER, M. T., JOHN, M., EMOND, R. T. D. & MACHACEK, K. A. (1965). Acquisition of *Staphylococcus aureus* by patients in cubicles. *Br. med. J.* i, 1101.

REPORT (1960). Incidence of surgical wound infection in England and Wales. *Lancet* ii, 659.

SHOOTER, R. A., THOM, B. T., DUNKERLEY, D. R., TAYLOR, G. W., PARKER, M. T., JOHN, M. & RICHARDS, I. D. G. (1963). Pre-operative segregation of patients in a surgical ward. *Br. med. J.* ii, 1567.

WILLIAMS, R. E. O., NOBLE, W. C., JEVONS, M. P., LIDWELL, O. M., SHOOTER, R. A., WHITE, R. G., THOM, B. T. & TAYLOR, G. W. (1962). Isolation for the control of staphylococcal infection in surgical wards. *Br. med. J.* ii, 275.

J. Hyg., Camb. (1966), **64**. 339
Printed in Great Britain

Sensitivity of bluetongue virus to lipid solvents, trypsin and pH changes and its serological relationship to arboviruses*

By S-E. SVEHAG†

Department of Veterinary Science, Washington State University Pullman, Washington

LOUISE LEENDERTSEN and J. R. GORHAM

The Animal Disease Research Station, Animal Disease and Parasite Research Division, U.S. Department of Agriculture, Pullman, Washington

(*Received* 29 *January* 1966)

Arboviruses are considered to have the following properties in common: they contain RNA and are sensitive to lipid solvents, biologic transmission by a vector is obligatory and they are pathogenic for Swiss mice by the intracerebral route. There is cogent evidence that bluetongue virus (BTV) is transmitted by arthropods (Du Toit, 1944; Foster, Jones & McCrory, 1963; Price & Hardy, 1954) and biological transmission is probable (Foster *et al.* 1963). The nucleic acid of BTV is believed to be RNA (Livingstone & Moore, 1962; Svehag, 1963) and the virus is pathogenic for mice by the intracerebral route (Svehag, 1962). For these reasons BTV has been tacitly classified as an arbovirus. However, BTV has not been placed in any of the described serological groups of arboviruses (Andrewes, 1964; Casals, 1961). Further, BTV possesses two characteristics which are unique for members of the arbovirus group, viz. its recently reported insensitivity to ether treatment (Studdert, 1965) and the fact that it is transmitted by gnats of the genus *Culicoides* (Diptera: *Ceratopogonidae*). This latter property it shares with African horse sickness virus which, like BTV is classified among 'ungrouped' arboviruses (Casals, 1961).

As physical and chemical properties in combination with antigenic relationships serve as more precise criteria for virus classification than pathogenicity and tissue affinity, the sensitivity of BTV to various chemical treatments was studied. The stability of BTV to lipid solvents such as ether or sodium deoxycholate (SDC) was confirmed and this observation extended to chloroform, a solvent of greater polarity. Cheng (1958) reported that trypsin inactivated arboviruses of the serological group B, while viruses in group A were unaffected. As this finding suggested that treatment with proteases can aid in the classification of arboviruses, the effect of trypsin on BTV was examined. The utility of exposure to low pH for the classi-

* Washington Agricultural Experiment Station, Scientific Paper no. 2773.

† Fellow of the American Scandinavian Foundation. Present address: Department of Virus Research, Karolinska Institutet Medical School, Stockholm, Sweden.

fication of virus strains is well illustrated by the picorna virus group, all enteroviruses being resistant to low pH while rhinoviruses rapidly lose their infectivity below pH 5·0–6·0. This treatment, useful as a preliminary step in virus identification, was also applied to BTV.

MATERIALS AND METHODS

Virus

A strain (BT-8) of bluetongue virus recovered from infected sheep in California (McKercher, McGowan & Saito, 1954) and originally passaged by yolk sac inoculation of chicken embryos was employed. BT-8 at the 180th embryo passage was adapted to the intracerebral route in unweaned mice (Svehag, 1962). Stock virus, at the 53rd mouse passage, was prepared in Tris buffer, pH 7·3 containing 200 i.u. penicillin and 100 μg. streptomycin per ml. The suspension was clarified by centrifugation at 3000 rev./min. for 20 min., pelleted by centrifugation at 30,000 rev./min. for 3 hr. and the pellet dissolved in Tris buffer and stored at $-70°$ C. in sealed glass ampoules.

The 65th passage of the Wisconsin FXNO strain of distemper virus (DV) was propagated in chicken embryos by the stab method (Gorham, 1957). Stock virus was prepared from infected chorioallantoic membranes in phosphate buffer, pH 7·4 containing 5% glycerine, and 50 μg. dihydrostreptomycin sulphate and 200 i.u. penicillin G potassium per ml. The suspension was clarified by low speed centrifugation and the virus stock stored at $-70°$ C. in sealed glass ampoules.

Infectivity assay

BTV was serially diluted five-fold or ten-fold and assayed intracerebrally (0·02 ml.) in 3-day-old unweaned Webster Swiss albino mice using 7–8 mice per dilution. Virus titres were expressed in $\log_{10}$ MLD 50 per ml. undiluted mouse brain homogenate. DV was titrated in embryonated eggs by the stab method (0·2 ml.), using five-fold dilutions and five chorioallantoic membranes per dilution. Virus titres were expressed in $\log_{10}$ EID 50 per ml. undiluted chorioallantoic membrane homogenate.

Neutralization tests

Two thousand LD 50 (0·2 ml.) of virus was mixed with 1·8 ml. antiserum; the reaction mixture was shaken and incubated at 37° C. for 24 hr. After incubation the virus-serum mixture was placed in an ice bath, and titrated for residual infectivity using two-fold serial dilutions.

Treatment with lipid solvents

Two ml. BTV preparation and 0·5 ml. diethyl ether (20%) were placed in air-tight tubes, shaken and incubated at 5° C. for 24 hr. during which time the tubes were intermittently shaken. Virus controls devoid of ether were similarly treated. After incubation the ether was removed and residual ether allowed to evaporate. The samples were then diluted in Tris buffer and assayed for infectivity.

One ml. BTV or DV preparation and 0·05 ml. chloroform were intermittently

shaken for either 10 min. at room temperature, 10 min. at 5° C. or 24 hr. at 5° C. The mixtures and virus controls were then centrifuged at 400 rev./min. for 5 min. and the clear top phase recovered and titrated for infectivity.

One ml. BTV preparation and 1 ml. SDC (1/500) in Tris buffer, pH 7·3 were mixed and incubated for 1 or 2 hr. at 37° C. These mixtures and virus controls, in which the Tris buffer replaced deoxycholate, were then assayed for infectivity.

Treatment with trypsin

Two different trypsin preparations were used: Difco Bacto trypsin (1/250) once crystallized and a trypsin preparation twice crystallized and salt free (Worthington Biochemical Corp., Freehold, New Jersey). The concentration of trypsin used varied between 0·05 and 20 mg./ml. The soybean trypsin inhibitor, 5 times crystallized (Worthington Biochemical Corp.) was employed at a concentration of 8 mg./ml. Both trypsin and the soybean trypsin inhibitor were dissolved in phosphate buffer, pH 8·0. Fresh trypsin solutions were prepared before each test since the trypsin gradually lost its activity in phosphate buffer at 4° C. For details concerning the experimental design see legend to Fig. 3.

RESULTS

Sensitivity to lipid solvents

BTV was found to be rather resistant to treatment with both ether, chloroform and SDC (Table 1). Treatment with chloroform was preferred to ether as the chloroform could be readily separated from the virus suspension by low-speed centrifugation while the ether had to be removed by evaporation. It appeared to be immaterial whether the virus preparation was incubated and shaken with chloroform for 10 min. or 24 hr. DV was used as a chloroform sensitive control virus.

Table 1. *Effect of lipid solvents on bluetongue virus*

Experiment	Incubation conditions	Treated	Control	No tests
BTV* + 0·1 % SDC	1 hr., 37° C.	7·0†	7·3	5
	2 hr., 37° C.	7·0	7·6	1
BTV‡ + ethyl ether	24 hr., 5° C.	8·0	8·3	3
BTV‡ + chloroform	10 min., 5° C.	7·0	7·6	2
	10 min., room temp.	7·4	8·1	2
	24 hr., 5° C.	7·5	8·3	1
DV‡ + chloroform	10 min., room temp.	0	4·5	2

* Final virus dilution $10^{-1.0}$. † Expressed as $\log_{10}$ LD50/ml.
‡ Final virus dilution $10^{-0.7}$.

Sensitivity to pH *changes*

The data in Fig. 1 show that BTV had a narrow zone of pH stability in Michaelis buffer between pH 6 and 8 similar to that of rhinoviruses and foot-and-mouth disease virus which also are RNA-containing and ether-resistant viruses. This is in contrast to most picornaviruses which are stable between pH 2 and 9. Reoviruses are reported to be stable between pH 4 and 8 (Wallis, Smith & Melnick, 1964).

Like several arboviruses (Western equine encephalomyelitis (WEE), Japanese B encephalitis, St Louis encephalitis), BTV was slightly more stable at alkaline than at acid pH. Eastern equine encephalomyelitis (EEE), Western equine encephalomyelitis and Semliki Forest virus resemble BTV also in their sensitivity to acid pH, EEE and WEE being inactivated below pH 6·4 and Semliki Forest virus below pH 6 (Andrewes, 1964).

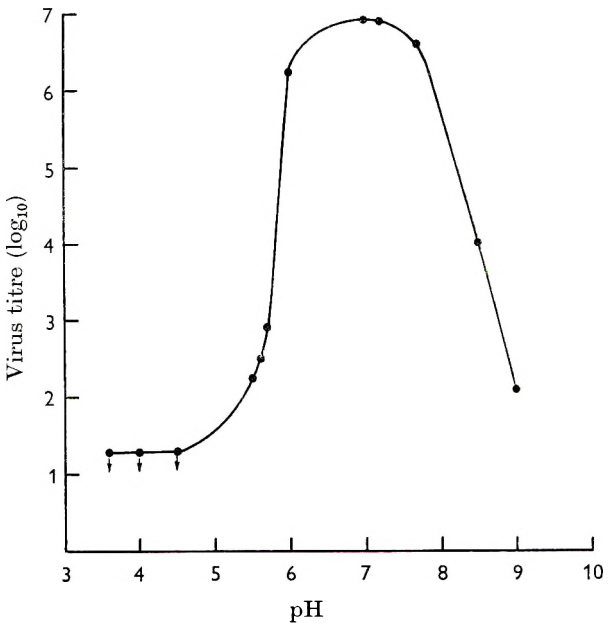


Fig. 1. Stability of BTV in Michaelis buffer at various pH values. 0·2 ml. BTV preparation (1/5) was mixed with 1·8 ml. Michaelis buffer and the pH of the mixture readjusted to neutrality with 0·1 N-NaOH after 1 min. incubation at 37 °C.; pH determinations were made on the BTV–buffer mixtures.

The inactivation of BTV within 1 min. below pH 6 and above pH 8 rendered it difficult to study the kinetics of the inactivation reaction in these pH regions. A pH (6·1) in the critical region was therefore chosen for a study of the reaction kinetics (Fig. 2). The kinetic curve had two components, about 90 % of the virus being inactivated rapidly while the residual infectivity was lost at a much reduced rate. A minor fraction of the virus population ($< 0{\cdot}01$ %) was rather resistant at this pH. This may be due to a protective effect from the clumping of virus particles or from extraneous protein in the virus preparation.

The pH inactivation appeared to be irreversible as the degree of inactivation was the same whether treated virus was assayed for infectivity immediately or several hours after adjustment of pH to neutrality.

Sensitivity to trypsin treatment

In the present study BTV was found to be inactivated to about 90 % by relatively low trypsin concentrations (50–100 μg/ml.) and the degree of inactivation to be proportional to the enzyme concentration (Fig. 3). The effect was not due to the

presence of nucleases or other contaminating active principles in the trypsin preparation as (1) two trypsin preparations of different purity gave identical results, and (2) the trypsin effect was completely abolished by preincubation of the trypsin with soybean trypsin inhibitor (8 mg./ml.), which is known to block only the active proteolytic part of the trypsin molecule (Green, 1953; Kunitz, 1947). It was also

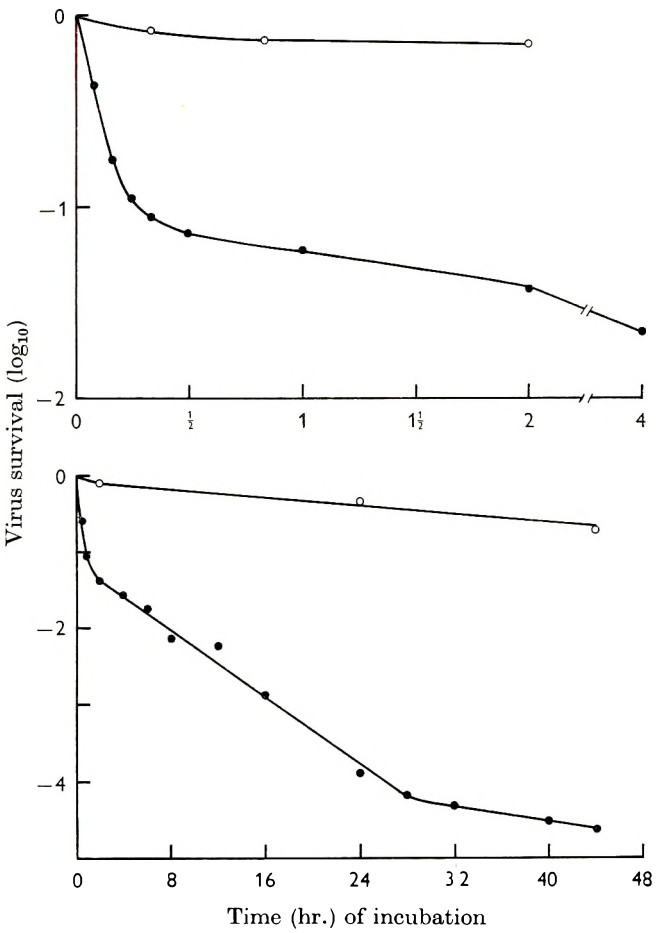


Fig. 2. Kinetics of the inactivation of BTV infectivity in Michaelis buffer at 37° C. 0·2 ml. BTV preparation (1/5) and 1·8 ml. Michaelis buffer, pH 6·1, ● ; 0·2 ml. BTV preparation and 1·8 ml. Tris buffer, pH 7·3, ○ ; pH determinations were made on the BTV–buffer mixtures. The points represent the geometric means of two tests.

demonstrated that trypsin-inactivated BTV was not reactivated by the addition of the trypsin inhibitor. These results indicated that the inactivation occurred via proteolysis of the viral particle and not simply by binding to virus receptors. Soybean trypsin inhibitor alone did not inactivate BTV.

It was unlikely that the trypsin effect was mediated by degradation products of brain tissue as the degree of BTV inactivation was diminished rather than increased when crude BTV preparations were used.

Serological classification

The grouping of arboviruses is based solely on immunological cross-reactions. The haemagglutination-inhibition test provides the broadest spectrum of antigenic overlap among these viruses, while the intracerebral neutralization test, in general, is more specific. Casals (1961) and Clarke (1960) have improved and extensively employed the former test for classification of arboviruses and Casals has divided arboviruses into four antigenic groups designated A, B, C and Bunyamwera groups. Remaining arboviruses are classified as 'ungrouped' and BTV is included in this group (Casals, 1961).

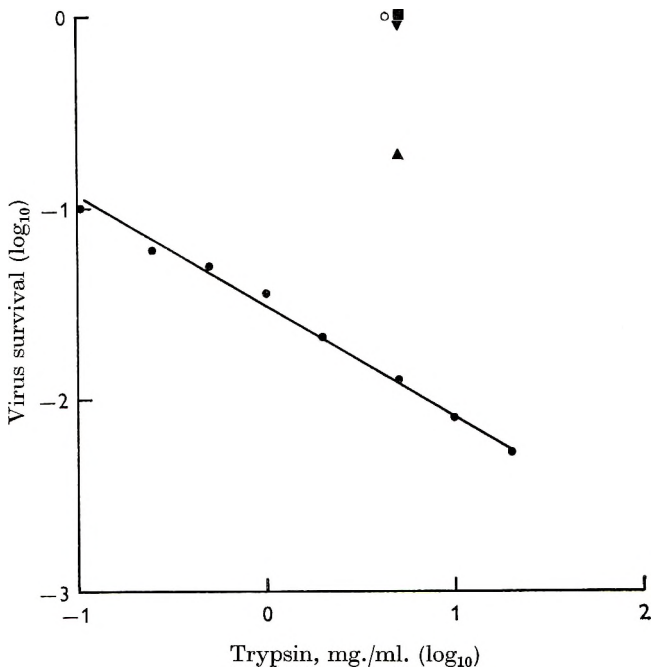


Fig. 3. Effect of trypsin and soybean trypsin inhibitor on BTV infectivity; 0·2 ml. BTV preparation (1/5) was mixed with either 1·8 ml. trypsin solution, ●; 1·8 ml. trypsin solution heated at 56° C. for 30 min., ▲; 1·8 ml. trypsin solution containing 15 mg. soybean trypsin inhibitor, ■; 1·8 ml. phosphate buffer containing 15 mg. soybean trypsin inhibitor, ▼, or 1·8 ml. phosphate buffer, pH 8·0, ○. All mixtures were incubated for 24 hr. at 37° C. before assay for infectivity. The points represent the geometric means of two to three tests.

The finding that BTV like arboviruses in group B was inactivated by trypsin led us to re-examine the possibility that an immunological cross-reaction existed between BTV and viruses belonging to this group. Virus neutralization, employing the immuno-inactivation technique (Gard, 1957) and the intracerebral route in unweaned mice was used. Immune mouse sera,* the lowest dilution tested being 1/10, prepared against St Louis, West Nile and Japanese B viruses (group B) were, however, found to exert no greater neutralizing effect on BTV than normal mouse

* Kindly supplied by Dr J. Casals, The Rockefeller Institute, New York.

serum. Similar results were obtained when immune mouse sera produced with EEE-85, Sindbis, Chikungunya viruses (group A), Carparu and Oriboca viruses (groups C) and Bunyamwera, Chittoor and Illesha viruses (the Bunyamwera group) were assayed against BTV. It should be emphasized, however, that these results require confirmation with the hemagglutination-inhibition test because of its greater sensitivity for the detection of cross-reactions.

DISCUSSION

All arboviruses so far examined have been sensitive to ether or SDC and this property has served to distinguish them from viruses such as picorna- and adenoviruses. The sensitivity of arboviruses to lipid solvents and the finding that RNA can be extracted from these viruses only by the 'hot phenol' method (Wecker, 1959) suggest that lipids are contained in an envelope which protects the RNA. Electron micrographs of WEE virus (Morgan, Howe & Rose, 1961) and Sindbis virus (Mussgay & Rott, 1964) have also indicated the existence of such an envelope. Myxoviruses and viruses belonging to the herpes group, which also are sensitive to lipid solvents, are all known to possess an envelope. The stability of BTV to lipid solvents and its sensitivity to trypsin indicates that this virus, in contrast to other arboviruses, is lacking a lipid-containing envelope but has a surface protein which interacts with the host cell. In a recent communication, Studdert (1965) states that unpublished electron micrographs suggest that BTV is devoid of an envelope.

Determination of the effect of enzymic action may provide useful information for the classification of virus groups (Gresser & Enders, 1961). As early as 1936 Merrill reported that EEE virus, belonging to group A arboviruses, was resistant to trypsin, and Cheng (1958) recently found this property to be shared by all group A arboviruses, while in contrast group B arboviruses were digestible by trypsin. In the present study, BTV was found to be trypsin sensitive but attempts to demonstrate a serological relationship between this virus and representative group B arboviruses failed.

The present results do not support the inclusion of BTV in the arbovirus group. It is instead becoming evident that BTV shares many properties with reoviruses. Both viruses contain RNA, resist treatment with lipid solvents (Gomatos, Tamm, Dales & Franklin, 1962) and are trypsin sensitive (Gomatos & Tamm, 1962; Mayor & Jordan, 1965). Of interest in this context are results by Polson & Deeks (1963) indicating that African horse sickness virus, another serologically unclassified, RNA-containing and ether-resistant arbovirus, which also is unstable below pH 6 and transmitted by *Culicoides* spp., has an ultrastructure which closely resembles that of reoviruses (Vasques & Tournier, 1962; Jordan & Mayor, 1962).

SUMMARY

Bluetongue virus was found to be resistant to ether, chloroform and sodium deoxycholate under a variety of conditions but sensitive to treatment with trypsin. The virus had a narrow zone of pH stability between pH 6 and 8 in Michaelis

buffer. Below pH 6 bluetongue was irreversibly inactivated within 1 min. at 37° C. In many of its characteristics, bluetongue virus appears to be closely related to the reoviruses.

REFERENCES

ANDREWS, C. H. (1964). *Viruses of Vertebrates*. London: Baillière, Tindall and Cox.

CASALS, J. (1961). Procedures for identification of arthropod-borne viruses. *Bull. Wld Hlth Org.* **24**, 723.

CHENG, P-Y. (1958). The inactivation of group B arthropod-borne animal viruses by proteases. *Virology* **6**, 129.

CLARKE, D. H. (1960). Antigenic analysis of certain group B arthropod-borne viruses by antibody absorption. *J. exp. Med.* **111**, 21.

DU TOIT, R. M. (1944). The transmission of blue-tongue and horse sickness by *Culicoides*. *Onderstepoort. J. vet. Sci. Anim. Ind.* **19**, 7.

FOSTER, N. M., JONES, R. H. & MCCRORY, B. R. (1963). Preliminary investigations on insect transmission of bluetongue virus in sheep. *Am. J. vet. Res.* **24**, 1195.

GARD, S. (1957). Immuno-inactivation of poliovirus. *Arch. ges. Virusforsch.* **7**, 449.

GOMATOS, P. J. & TAMM, I. (1962). Reactive sites of reovirus type 3 and their interaction with receptor substances. *Virology* **17**, 455.

GOMATOS, P. J., TAMM, I. DALES, S. & FRANKLIN, R. M. (1962). Reovirus type 3: physical characteristics and interaction with L cells. *Virology* **17**, 441.

GORHAM, J. R. (1957). A simple technique for the inoculation of the chorio-allantoic membrane of chicken embryos. *Am. J. vet. Res.* **18**, 691.

GREEN, N. M. (1953). Competition among trypsin inhibitors. *J. biol. Chem.* **205**, 535.

GRESSER, I. & ENDERS, J. F. (1961). The effect of trypsin on representative myxoviruses. *Virology* **13**, 420.

JORDAN, L. E. & MAYOR, H. D. (1962). The fine structure of reovirus, a new member of the icosahedral series. *Virology* **17**, 597.

KUNITZ, M. (1947). Crystalline soybean trypsin inhibitor. II. General properties. *J. gen. Physiol.* **30**, 291.

LIVINGSTONE, C. W. & MOORE, R. W. (1962). Cytochemical changes of bluetongue virus in tissue cultures. *Am. J. vet. Res.* **23**, 701.

MAYOR, H. D. & JORDAN, L. E. (1965). Studies on reovirus. I. Morphologic observations on the development of reovirus in tissue culture. *Expl molec. Path.* **4**, 40.

MCKERCHER, D. G., MCGOWAN, B. & SAITO, J. K. (1954). Studies on bluetongue. I. Isolation, identification and typing of the bluetongue virus and a preliminary report on the serodiagnosis of the disease. *Proc. Am. vet. med. Ass.* p. 167.

MERRILL, M. H. (1936). Effect of purified enzymes on viruses and Gram-negative bacteria. *J. exp. Med.* **64**, 19.

MORGAN, C., HOWE, C. & ROSE, H. M. (1961). Structure and development of viruses as observed in the electron microscope. V. Western equine encephalomyelitis virus. *J. exp. Med.* **113**, 219.

MUSSGAY, M. & ROTT, R. (1964). Studies on the structure of a hemagglutinating component of a group A arbo virus (Sindbis). *Virology* **23**, 573.

POLSON, A. & DEEKS, D. (1963). Electron microscopy of neurotropic African horse-sickness virus. *J. Hyg., Camb.*, **61**, 149.

PRICE, D. A. & HARDY, W. T. (1954). Isolation of the bluetongue virus from Texas sheep—Culicoides shown to be a vector. *J. Am. vet. med. Ass.* **124**, 255.

STUDDERT, M. J. (1965). Sensitivity of bluetongue virus to ether and sodium deoxycholate. *Proc. Soc. exp. Biol. Med.* **118**, 1006.

SVEHAG, S-E. (1962). Quantitative studies of blue tongue virus in mice. *Arch. ges. Virusforsch.* **12**, 363.

SVEHAG, S-E. (1963). Thermal inactivation of blue tongue virus. *Arch. ges. Virusforsch.* **13**, 499.

VASQUEZ, C. & TOURNIER, P. (1962). The morphology of reovirus. *Virology* **17**, 503.

WALLIS, C., SMITH, K. O. & MELNICK, J. L. (1964). Reovirus activation by heating and inactivation by cooling in $MgCl_2$ solutions. *Virology* **22**, 608.

WECKER, E. (1959). The extraction of infectious virus nucleic acid with hot phenol. *Virology* **7**, 241.

J. Hyg., Camb. (1966), **64**, 347
With 1 *plate*
Printed in Great Britain

The survival of *Toxoplasma* in infected mosquitoes

By JENNIFER M. CHAPMAN and W. E. ORMEROD

Department of Parasitology,
London School of Hygiene and Tropical Medicine,
Keppel Street, London, W.C. 1

(*Received* 7 *March* 1966)

INTRODUCTION

It has often been suggested that arthropods can act as transmitting agents for *Toxoplasma gondii* Nicolle & Manceaux, and the evidence has been reviewed by Jacobs (1953), with further experiments by Laarman (1956), Rifaat & Morsy (1962), Deane (1958) and Nussenzweig & Deane (1958).

The experiments described in this paper were suggested by the following circumstantial evidence that the mosquito *Culiseta annulata* Shrank might be acting as the transmitting agent for *Toxoplasma* infection from pigs to man in and around the town of Barton-upon-Humber, Lincolnshire, England. The Medical Officer of Health had noted the persistently high perinatal mortality for this area for the years 1956–59 (Robertson, 1960) and had suggested that this might be due to toxoplasmosis on account of the unusually high rate of positive reactions to the *Toxoplasma* dye test of Sabin & Feldman (1948). He had also noted that in the 2–4 years age group the conversion from positive to negative occurred during the late summer; this, he believed, might indicate a mosquito-borne infection since the population of mosquitoes was abnormally high.

In October 1961 one of us (W. E. O.) visited Barton-upon-Humber together with Mr P. G. Shute and Miss Maryon of the Malaria Reference Laboratory. Even at this late season very large numbers of larvae of *C. annulata* were found in flooded claypits, the water of which had been contaminated with town rubbish giving the high organic content required by this species.

Pig production is an important industry in the area both on intensive and back-yard scales; local veterinarians had diagnosed toxoplasmosis in pigs and this had been confirmed by the dye test.

There were many piggeries both in and around the town from which the following mosquitoes, of species known to bite man, were collected: 54 *Culiseta annulata*, 21 *Anopheles claviger* Meigen, 1 *A. labranchiae atroparvus* van Thiel.

These were ground up in batches of one to nine mosquitoes and injected into multimammate mice, *Mastomys coucha*, which were killed 5 weeks later and dye tests performed by Dr Fleck of Swansea. Experimental and control animals showed titres up to 1/8, but one experimental *Mastomys* into which seven *Culiseta* from a piggery in Barton-upon-Humber had been injected, showed a titre of 1/128. Despite prolonged searching of sections of the brain of this *Mastomys* no *Toxoplasma* cysts or free forms were seen.

The piggery in question had been abandoned shortly after the experiment because of unexplained losses and lack of fertility in the stock.

Next year ecological conditions had changed. As a result of late frosts, the massive growth of population of *Culiseta* that had appeared in previous years did not occur and a repetition of the abortive field experiments described above was not feasible. We therefore decided to investigate under laboratory conditions the fate of *Toxoplasma* free forms and cysts in *Culiseta annulata* and other mosquitoes, and in this way to obtain information on the susceptibility of mosquitoes to infection and their possible ability to act as transmitting agents for *Toxoplasma* in the field.

MATERIALS AND METHODS

Strain of Toxoplasma

Most previous experiments on the transmission of *Toxoplasma* by arthropods have been conducted using the acute RH strain of the organism. It seemed, however, unlikely that this type of strain would be involved in the relatively benign infections which occurred at Barton-upon-Humber. In the present series of experiments, therefore, we used an avirulent or chronic strain previously isolated from sheep during a survey of the East Midlands and Yorkshire (Beverley & Mackay, 1962; Beverley & Watson, 1961), kindly supplied to us by Dr Beverley. A chronic strain such as this, which on passage goes through more of its life-cycle, seems more likely to be transmissible by a vector than the RH strain which has undergone serial passage for many years involving only the trophozoite form.

Mice infected intraperitoneally with cysts of the Beverley strain produce only a slight peritoneal exudate; a few trophozoites can be seen in Giesma stained preparations from peritoneal fluid during the first 14 days but after this time they disappear and cannot be demonstrated by direct microscopy. This phase of multiplication is usually accompanied by a low level of parasitaemia. Remington, Melton & Jacobs (1961) showed that this was of short duration and was followed by localization and encystment in the brain and other organs. Cysts are detectable in the homogenate of brain material from about 14 days after infection.

The majority of animals do not show symptoms of disease but about 5% succumb. In these, 10 days after infection, the abdomen is distended with peritoneal exudate containing intra- and extracellular trophozoites.

For routine passage, 20 g. white mice obtained from Messrs Tucks each received an inoculum of approximately 20 cysts by the intraperitoneal route. Counting was performed on 0·01 ml. brain homogenate under a coverslip using phase contrast at $\times 100$ magnification.

The time interval between successive passages was about 3 months.

Production of maximum numbers of trophozoites

Mastomys were inoculated intraperitoneally with 0·01 ml. of a suspension of hydrocortisone acetate containing 250 mg./ml. (Hydrocortistab, Boots), 5 days before infection with about 300 cysts of *Toxoplasma*. On the 7th-8th day after infection there were at least six intracellular trophozoites per ml. On the ninth day the animals died with characteristic symptoms.

Production of the maximum numbers of cyst forms

Mice (15 g.) were inoculated intraperitoneally each with about 150 cysts. This resulted in loss of about 40 % of the mice between the fifth and eleventh day following infection, and the remainder failed to develop in weight and size as rapidly as control mice of the same age. Brains of these infected mice examined from the tenth day onwards showed large numbers of cysts, which were small at first, ranging from about 7 to 10 μ in diameter and containing only a few organisms, and reached about 40 μ in diameter at 4 weeks. In these mice the concentration of cysts varied between 30 and 40 per 0·01 ml. sample of a homogenate of the whole brain in 1 ml. of saline.

It was also found that passage of the Beverley strain from the brains of infected laboratory mice into cotton rats (*Sigmodon*) increased the yield of cysts obtained on repassage to laboratory mice. The concentration from rat brain homogenate was found to be two to three cysts per 0·01 ml., and when this was repassaged intraperitoneally into 15 g. mice at the rate of 70–80 cysts per mouse, a final concentration of 40–50 cysts per 0·01 ml. sample of brain homogenate was obtained with similar mortality among the mice. This is equivalent to about double the yield of cysts using laboratory mice only.

Strains of mosquitoes

Experiments were carried out using four species of mosquitoes. Laboratory cultures of *Aedes aegypti*, *A. togoi* and *Anopheles labranchiae atroparvus* were available in the Department of Entomology at this School. A culture of *C. annulata* was established for use in these experiments from a collection of gravid females made at Barton-upon-Humber. Multiplication of the colony depended upon the use of artificial insemination.

Infection of mosquitoes and subsequent maintenance

The mosquitoes were fed on infected material through a membrane consisting of the skin of a freshly killed mouse, which was stretched over the bottom of a glass tube, 1·8 cm. in diameter. The bottom of the tube had been pressed in to form a concavity which contained the infective or control material on which the mosquitoes were to be fed. The tube contained water at 37° C., heated and made to circulate with a 'Circotherm' unit. The mouse skin rested on the top of a 4·5 in. cubical cage containing about 50 experimental mosquitoes. The apparatus was set up at air temperature of 20° C. for *C. annulata* and 25–26° C. for the other species.

Feeding was allowed to continue for about 1 hr., after which the fed females were separated by placing the cage in a totally enclosed transparent cabinet and transferring them by mechanical suction to another similar cage. The apparatus was constructed in such a way that there was no possibility of an infected mosquito escaping into the laboratory.

Two or three mosquitoes from each cage were ground up in saline and injected with 20,000 units of penicillin into mice daily for about 14 days. In some instances mosquitoes were fixed in Carnoy's solution, 'jellified' in benzene and embedded in

wax for sectioning. Sections of 4 μ were stained by the Giemsa-Colophonium method of Shortt & Cooper (1948). Homogenates of the brains of injected mice were examined after 1 month and if no cysts were observed, a further passage was made and the brain homogenate re-examined after another month. Stained smears and sections were also examined; they were fixed in Bouin's fluid and studied by the method of Lainson (1955).

The viability of toxoplasms in the feed was tested at the end of each feeding period by injection into mice.

In most experiments (see Tables 1 and 2) blood was added to the infective material. This was to ensure the passage of the infective material into the insect's stomach. In some instances blood was omitted, to test the survival of toxoplasms in the insect crop where the feed is partially retained in the absence of blood, as it was thought that toxoplasms remaining in the crop of a mosquito and not subjected to the digestive juices of the stomach might remain infective for longer.

In addition to the experiments carried out by membrane feeding *C. annulata* was also induced to feed directly on a mixture of homogenized brain and 10% sucrose. Controls showed that viability was not affected by this treatment. Since regurgitation from the crop under these conditions might be expected to occur, the mosquitoes were allowed to feed on baby mice which were examined for cysts in the brain after 1 month.

RESULTS

Results of experiments to infect mosquitoes with Toxoplasma *and of attempts to transmit the disease*

The survival of trophozoites in four species of mosquito which had been fed on infected material through a membrane was shown by inoculation of ground-up mosquitoes into mice to vary within narrow limits (see Table 1). Counting the day of feeding as the first day, viable trophozoites were present only on the first day in *Aedes togoi* and *Anopheles labranchiae atroparvus*, up to the second day in *Culiseta annulata*, and up to the third day in *Aedes aegypti*. These figures suggest that trophozoites remain viable as long as the blood meal remains undigested, and also that the different temperatures at which the cultures of fed mosquitoes were held did not affect their survival time.

Microscopic examination of the Giemsa-stained smears and sections of brain material of recipient mice failed to reveal cases of the infection which had not already been detected by examination of fresh material.

A number of cyst-like bodies were seen from time to time in fresh preparations, but because of the slight difference in their structure from true cyts of *Toxoplasma*, their random appearance in both control and experimental animals, their appearance only in fresh, never in stained preparations, and their failure to produce infection on inoculation into clean mice, we conclude that they were unrelated to *Toxoplasma*. (Similar bodies were observed by Dr Om Prakash while working in this laboratory.) One such body, found in brain material taken from a mouse inoculated with a number of ground-up *Aedes aegypti* is shown in Plate I. This was at first thought to be a cyst of *Toxoplasma*, as it was of a comparable size, but no

Table 1. *Summary of results of experiments to infect mosquitoes with trophozoite-forms of* Toxoplasma

Species of mosquito	No. of times experiment repeated	Presence of other fluid 1:1 ratio	Temperature of mosquito culture °C	Control	Interval (days) between infection of mosquito and injection of mosquito suspension into mouse, and presence or absence of cysts in the brain of that mouse 1 month later													
					1	2	3	4	5	6	7	8	9	10	11	12	13	14
Aedes aegypti	5	Blood	26	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
			37		+	+	+	−	−	−	−	−	−	−	−	−	−	−
			(26)		+	+	+	−	−	−	−	−	−	−	−	−	−	−
			(37)		+	+	+	−	−	−	−	−	−	−	−	−	−	−
Aedes togoi	2	Blood	26	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
			37		+	−	−	−	−	−	−	−	−	−	−	−	−	−
			(26)		+	−	−	−	−	−	−	−	−	−	−	−	−	−
			(37)		+	−	−	−	−	−	−	−	−	−	−	−	−	−
Anopheles l. atroparvus	4	Blood	26	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
			37		+	+	−	−	−	−	−	−	−	−	−	−	−	−
			(26)		+	+	−	−	−	−	−	−	−	−	−	−	−	−
			(37)		+	+	−	−	−	−	−	−	−	−	−	−	−	−
Anopheles l. atroparvus	1	—	26	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
			37		+	+	−	−	−	−	−	−	−	−	−	−	−	−
Culiseta annulata	3	Blood	20	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
					+	+	−	−	−	−	−	−	−	−	−	−	−	−
					+	+	−	−	−	−	−	−	−	−	−	−	−	−
					+	+	−	−	−	−	−	−	−	−	−	−	−	−

1st passage

2nd passage

1st passage

2nd passage

1st passage

2nd passage

1st passage

2nd passage

In several cases mice died before examination for cysts was possible. These deaths are not recorded in the table, as parallel experiments covered such instances

Key: +, Cysts found in mouse brain when examined microscopically 1 month after inoculation with mosquitoes. −, No cysts found. Control: the viability of toxoplasms in the feed at the time of experiment was tested by inoculating mice with a sample of the feed.

Table 2. *Summary of results of experiments to infect* Culiseta annulata *with cyst-forms of* Toxoplasma gondii

Species of mosquito	No. of times experiment repeated	Presence of other fluid 1:1 ratio	Temperature of mosquito culture °C	Control	Interval (days) between infection of mosquito and injection of mosquito suspension into mouse, and presence or absence of cysts in the brain of that mouse 1 month later														
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	
C. annulata	3	Blood	20	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	1st passage
			26		+	+	–	–	–	–	–	–	–	–	–	–	–	–	
					+	+	–	–	–	–	–	–	–	–	–	–	–	–	2nd passage
					+	+	–	–	–	–	–	–	–	–	–	–	–	–	
C. annulata	3	10 % sucrose	20	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	1st passage
					+	+	+	–	–	–	–	–	–	–	–	–	–	–	2nd passage

Key: +, Cysts found in mouse brain, when examined microscopically, 1 month after inoculation with mosquitoes, or 1st passage mouse brain. –, No cysts found. Control: the viability of toxoplasms in the feed at the time of experiment was tested by inoculating mice with a sample of the feed.

disease was produced in two passages of this material through mice. As experience was obtained it became apparent that the wall of this 'cyst' was penetrated by several pores, and that the 'trophozoites' had a honeycomb appearance which became particularly obvious in partly dried preparations, as in Plate 1, fig. 2. Although we were uncertain of the nature of this body it seemed unlikely to be a parasitic protozoon.

Stained sections of mosquitoes, when examined under the microscope, did not reveal any sign of parasites, possibly because it was often difficult to obtain a good section of the blood meal within the mosquito stomach.

The experiment in which *A. l. atroparvus* were fed on a blood-free meal of infective trophozoites and then injected into mice showed that infective *Toxoplasma* were retained up to the second day only.

There was no evidence of transmission of the disease to baby mice on which infected mosquitoes had been allowed to feed.

Results of experiments to infect Culiseta annulata *with cyst-forms of* Toxoplasma gondii, *and to determine parasite survival time*

The survival of viable *Toxoplasma* in *C. annulata* fed on infected brain containing cysts and 10% sucrose was shown, by mouse inoculation, to last up to the third day, but when mixed with blood the parasites remained viable only up to the second day (Table 2).

All attempts to transmit the infection to anaesthetized baby mice, by allowing artificially infected mosquitoes to feed on them, were unsuccessful.

Careful examination of Giemsa stained crush-smears of crops dissected from mosquitoes which had fed on the infected brain-sucrose mixtures showed no cysts.

DISCUSSION

In the inconclusive field study which suggested the experiments recorded in this paper, it appeared that the *Culiseta annulata*, which was present in exceptionally large numbers at Barton-upon-Humber, might be connected with the high rate of dye-test antibodies in man; we therefore wished to investigate the possibility that *Toxoplasma* taken up in the mosquitoes' feed might be transmissible to another host. We found that the low roofs of pigsties where the mosquitoes rested were often very hot and we felt that this might be a factor in making the mosquitoes infective. In fact we could not demonstrate any effect of temperature, although this may have been because the mosquitoes did not live long enough under the experimental conditions.

Mosquitoes might become infected in the field in two ways: by taking trophozoites in blood or lymph from an acutely infected animal or by taking up cysts while probing the subcutaneous tissue of an animal harbouring a chronic infection, and the organisms obtained in either way might lodge in the crop or in the stomach of the mosquito. Although it is most unlikely that under natural conditions as high a concentration of organisms could be obtained in the infective feed as we achieved in our experiments, yet some degree of concentration of organisms might result by

repeated feeding on infected animals. The ability of a mosquito under these conditions to become an efficient transmitting agent presupposes that the infective organism develops in it and this we have been unable to demonstrate.

We have, however, demonstrated conclusively that a mosquito can retain an infection with cysts or trophozoites up to the third day from the time of infection and that it is not infective after this. We cannot exclude the possibility that an occult form is present and that it becomes infective after the fourteenth day.

We do not think that transient infectiveness of mosquitoes is likely to be a major contribution to the spread of toxoplasmosis, the more since the bite of mosquitoes during this period has not been shown to be infective; especially since the survival time of the organism is less than the normal interval between successive feeds in nature. It is necessary either to inject the mosquito or at least to crush it into a wound to obtain transmission. Yet, despite these limitations, so frequently is the organism found in nature that it is not unlikely that infection in man can occasionally occur by this means, i.e. by the crushing of an infected mosquito into a skin abrasion.

SUMMARY

1. An incomplete field study in Lincolnshire, England, suggested that toxoplasmosis might be transmitted from pig to man by mosquitoes. Although the results were inconclusive they suggested the experimental work recorded in this paper.

2. Trophozoites of the Beverley strain were obtained in increased yield from the peritoneal fluid of *Mastomys* treated with hydrocortisone. Cysts were obtained in increased yield by passage of the brain of *Sigmodon* into laboratory mice.

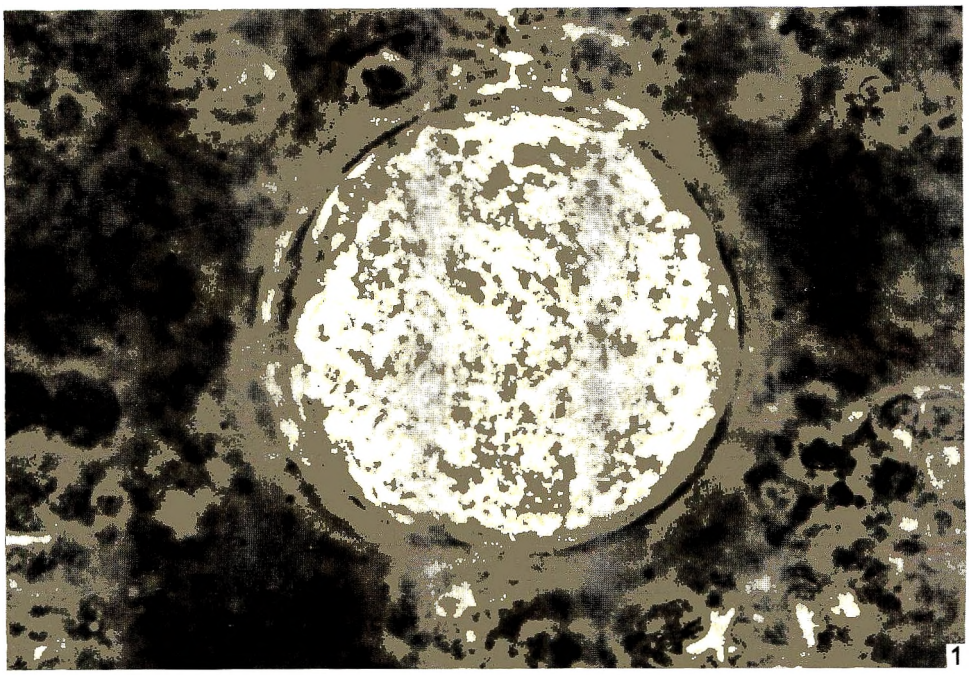
3. Four species of mosquitoes were fed through a membrane or on sucrose solution on media containing either cysts or trophozoites of *Toxoplasma gondii*. In some experiments blood was added to ensure passage of the feed to the mosquito stomach, in others it was excluded so that at least part would pass to the crop.

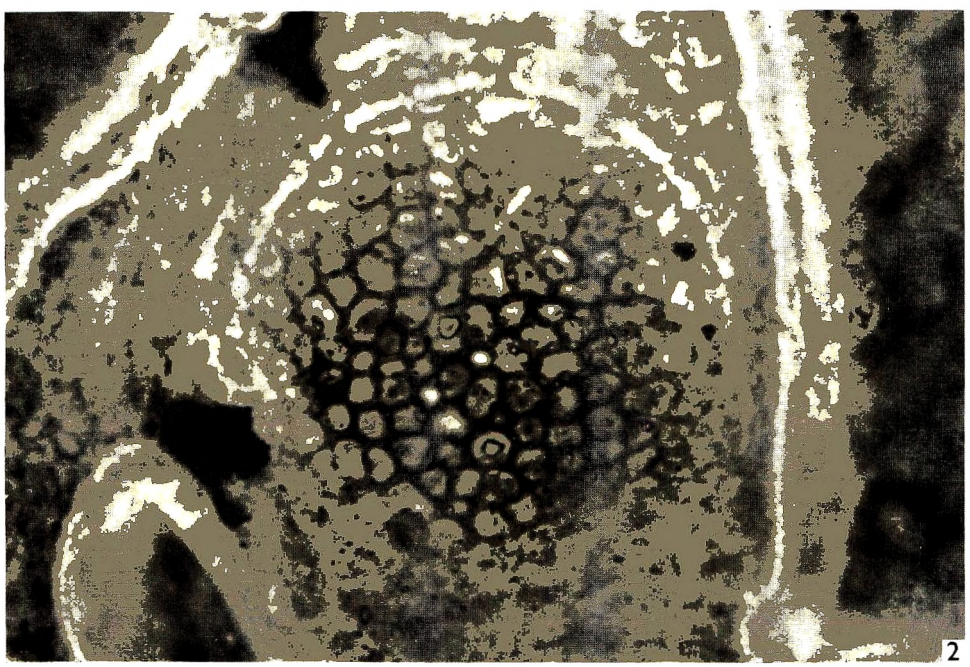
4. A spurious 'cyst' resembling but distinguishable from *Toxoplasma* was noted in the homogenate of mouse brain.

5. In no instance did mosquitoes retain infectivity as demonstrated by injection into mice, beyond the third day, but since experiments were not carried on beyond the fourteenth day the existence of an occult form cannot be excluded.

We would like to thank Dr J. S. Robertson, Prof. D. S. Bertram and Prof. P. C. C. Garnham for advice and practical help; also Mr M. J. Page for technical assistance.

The work was financed by the Medical Research Council.


1


2

REFERENCES

BEVERLEY, J. K. A. & MACKAY, R. R. (1962). Ovine abortion and toxoplasmosis in the East Midlands. *Vet. Rec.* **74**, 499.

BEVERLEY, J. K. A. & WATSON, W. A. (1961). Ovine abortion and toxoplasmosis in Yorkshire. *Vet. Rec.* **73**, 6.

DEANE, M. P. (1958). Estudos sobre transmissao do *Toxoplasma gondii*. II. Nota sobre a transmissao experimental pelo carrapato *Amblyomma cajennense*. *Revta bras. Malar. Doenç. trop.* **10**, 551.

JACOBS, L. (1953). The biology of *Toxoplasma*. *Am. J. trop. Med. Hyg.* **2**, 365.

LAARMAN, J. J. (1956). Transmission of experimental toxoplasmosis by *Stomoxys calcitrans*. *Documenta Med. geogr. trop.* **8**, 293.

LAINSON, R. (1955). Studies on the epidemiology of *Toxoplasma gondii* in England. The study of *Toxoplasma* in fixed and stained preparations. University of London Ph.D. Thesis vol. 1, p. 149).

NUSSENZWEIG, R. S. & DEANE, M. P. (1958). Estudos sobre transmissao do *Toxoplasma gondii*. I. Experiencias com triatomineos. *Revta bras. Malar. Doenç. trop.* **10**, 543.

REMINGTON, J. S., MELTON, M. L. & JACOBS, L. (1961). Induced and spontaneous recurrent parasitemia in chronic infections with avirulant strains of *Toxoplasma gondii*. *J. Immun.* **87**, 578.

RIFAAT, M. A. & MORSY, T. A. (1962). Experimental transmission of toxoplasmosis in laboratory animals and birds. *J. trop. Med. Hyg.* **65**, 314.

ROBERTSON, J. S. (1960). Excessive perinatal mortality in a small town associated with evidence of toxoplasmosis. *Br. med. J.* ii, 91.

SABIN, A. B. & FELDMAN, H. A. (1948). Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoon parasite (Toxoplasma). *Science, N.Y.*, **108**, 660.

SHORTT, H. E. & COOPER, W. (1948). Staining of microscopical sections containing protozoal parasites by modifications of McNamara's method. *Trans. R. Soc. trop. Med. Hyg.* **41**, 427.

EXPLANATION OF PLATE

Comparison of spurious 'toxoplasma cyst' with a cyst of *Toxoplasma gondii* of comparable size (60 μ across), both photographed under phase contrast with × 100 oil-immersion objective, from the homogenate of mouse brain.

Fig. 1. Cyst of *Toxoplasma* showing unbroken cyst wall and the pattern produced by internal trophozoites.

Fig. 2. Spurious 'cyst' showing honeycomb pattern (exaggerated by the preparation being partly dried).

J. Hyg., Camb. (1966), **64**, 357
Printed in Great Britain

The immunity produced by a rough *Salmonella dublin* variant against *Salmonella typhimurium* and *Salmonella choleraesuis* infection in guinea-pigs

By H. WILLIAMS SMITH and SHEILA HALLS
The Animal Health Trust, Stock, Essex

(*Received* 18 *February* 1966)

In a recent study (Smith, 1965) vaccination with a rough variant of *Salmonella dublin*, vaccine 51, produced a high degree of immunity in mice against oral infection with *S. typhimurium* and *S. choleraesuis*, the immunity being of the same order as that obtained against *S. dublin* itself. Vaccination with this variant did not protect against *Erysipelothrix rhusiopathiae* and *Escherichia coli* infection. However, since the vaccinal mortality in the mice was 33–45 %, it was felt that the protective effect against *S. typhimurium* and *S. choleraesuis* would be even more convincing if it could be demonstrated in an animal species in which vaccination had no apparent ill-effect. Such a demonstration is reported in this paper.

MATERIALS AND METHODS

Guinea-pigs

Young adult guinea-pigs of both sexes, weighing 400–480 g. were used. They were fed on Diet 18 (Oxoid) *ad lib.*

Vaccination

Three weeks before challenge vaccine 51, the rough variant of *S. dublin*, was administered subcutaneously as a single dose of 0·5 ml. of a 24 hr broth culture containing approximately 50×10^7 viable organisms per ml.

Method of challenge

After an overnight fast, guinea-pigs were given, by mouth, 0·25 ml. of an aqueous suspension of an 18 hr. nutrient agar culture of *S. typhimurium* or *S. choleraesuis* var *kunzendorf* to which was added a powder consisting of powdered chalk, 40 %; colloidal kaolin, 43 %; magnesium trisilicate 17 %. Each animal received approximately 5×10^8 viable organisms and 0·2 g. of powder. All those that died were examined for lesions, and the liver, spleen, small intestine and large intestine were examined by direct culture on deoxycholate-citrate agar to confirm that they had died from the particular serotype with which they had been challenged. The experiments were terminated 18 days after challenge when it was apparent from the condition of the guinea-pigs that no more would die.

The examination of faeces for salmonellas

Rectal swabs, taken so as to include a liberal portion of faeces, were cultured on deoxycholate-citrate-agar before and after enrichment in selenite-F medium for 24 hr. at 37° C. The plates were incubated for 24 hr. at 37° C. and then examined for the presence of salmonellas.

RESULTS

Ninety-eight guinea-pigs were used in all. Half of them were vaccinated with the rough *Salm. dublin* variant 51, and half were kept as controls. Four separate experiments were performed at different times, two with *S. typhimurium* as the challenge strain and two with *S. choleraesuis* as challenge strain; in each experiment the number of vaccinated and control guinea-pigs was the same.

Table 1. *The immunity conferred by* Salmonella dublin *vaccine* 51 *against challenge in guinea-pigs with* S. typhimurium *and* choleraesuis

	Challenge organism			
	S. typhimurium		*S. choleraesuis*	
	Vaccinated	Non-vaccinated	Vaccinated	Non-vaccinated
No. of guinea-pigs	22	22	27	27
No. that died after challenge	3	15	3	20
No. of survivors with liver or spleen lesions	1	6	4	4
No. of survivors in which salmonellas were found in liver or spleen*	0	3	3	3

* By direct culture on deoxycholate-citrate agar.

None of the guinea-pigs appeared unwell as a result of vaccination. On a general health basis the vaccinated groups were indistinguishable from the corresponding unvaccinated control groups. The food consumption of vaccinated and control groups was similar. Eighteen guinea-pigs were weighed immediately before vaccination and 17 days afterwards. On both occasions there was no difference between their weights and those of animals in the corresponding control groups.

The faeces of eleven guinea-pigs were examined 10 days after vaccination and those of another twelve, 3 and 7 days after vaccination. Salmonellas were not found in any of them or in any of the specimens from the corresponding control guinea-pigs.

The results of infecting the guinea-pigs are shown in Table 1; the vaccinated groups had a good immunity against *S. typhimurium* and *S. choleraesuis*.

DISCUSSION

The results confirm the observation made in mice that vaccination with a rough variant of *S. dublin* confers immunity against *S. typhimurium* and *S. choleraesuis*. They provide further evidence for the view that the antigens concerned with the

identification and serological classification of salmonellas are not involved in the immune process. Those that are involved can be common to members of different serological groups.

It is conceivable that the vaccine may have an application in guinea-pigs employed in long-term experiments, principally of a non-infectious nature, and kept under conditions that cannot exclude the possibility of the occurrence of an outbreak of natural salmonella infection.

SUMMARY

Vaccination with a rough variant of *Salmonella dublin* had no observable harmful effect on guinea-pigs. It conferred a good immunity against *S. typhimurium* and *S. choleraesuis* var *kunzendorf* infections.

REFERENCE

SMITH, H. WILLIAMS (1965). The immunization of mice, calves and pigs against *Salmonella dublin* and *Salmonella cholerae-suis* infections. *J. Hyg., Camb.* **63**, 117.

J. Hyg., Camb. (1966), **64**, 361
Printed in Great Britain

A method of secondary enrichment for salmonellas independent of selectively toxic chemicals

By R. W. S. HARVEY, D. E. MAHABIR and T. H. PRICE
Public Health Laboratory, Cardiff

(*Received* 23 *February* 1966)

INTRODUCTION

Man's environment is constantly contaminated with salmonellas and the technical problems of their isolation from heavily contaminated samples are of a different order from those confronting most hospital bacteriologists.

As a public health bacteriologist becomes more experienced, so his awareness increases of the danger of reporting false negative results. Bacteriology is, at best, an inexact science. Our attempts at selective culture of a particular micro-organism are still imperfect, even when well tried techniques are used.

Each attempt to improve the technique of salmonella isolation (Harvey & Thomson, 1953; Harvey, 1956; Harvey, 1957; Harvey & Price, 1962; Harvey & Price, 1964) has emphasized that a report 'failed to find salmonellas' was more truthful than the usual wording 'negative for salmonellas'. In an article on salmonella epidemiology, when the incidence of salmonella contamination of a material is given and conclusions are drawn from the results, it is well to remember that these same conclusions are only as valid as the figures on which they are based. It is in such investigations particularly that the bacteriologist requires to use the best available isolation techniques.

During the examination of crushed Indian bone for salmonellas (Harvey & Price, 1962), it was accidentally discovered that the passage of a mixed growth of salmonellas and other organisms through a modified Craigie tube (Harvey & Price, 1961), increased the ratio of salmonellas to other bacteria present. This was, therefore, a method of enrichment independent of selectively toxic chemicals. The phenomenon was not a new one. It recalled earlier attempts to isolate salmonellas from a mixed flora (Carnot & Garnier, 1902; Carnot & Weill Hallé, 1915; Friedburger, 1919; Friedburger & Putter, 1920; Pijper, 1952; Ino & Graber, 1955). The technique does not seem, however, to have been used extensively in routine practice, probably owing to the development of efficient enrichment media, such as tetrathionate and selenite broths.

The present paper is concerned with demonstrating that, occasionally, selective agars reported as negative for salmonellas by *experienced* observers are in actual fact positive. The method of demonstration employs the technique discussed above.

MATERIALS

The samples examined in this investigation were of two types; abattoir swabs and sewage polluted river water.

Abattoir swabs, in our laboratory, are incubated in selenite F broth at 43° C. and are subcultured to deoxycholate citrate agar, brilliant-green MacConkey agar (Harvey, 1956) and de Loureiro's (1942) modification of Wilson & Blair's medium. Incubation of highly contaminated samples in selenite F broth at 43° C. inhibits the growth of proteus species and is very useful for the isolation of salmonellas from sewage, bakery swabs and abattoir swabs (Harvey & Thomson, 1953; Harvey & Phillips, 1955; Harvey, 1956; Harvey, 1957; Harvey & Phillips, 1961). The three selective agars are incubated at 37° C and examined after 24–48 hr. incubation. It often happens that some but not all of these selective agars are positive, although inoculated from the same selenite F broth. We were interested in the *apparently* negative plates as suitable material for investigating the new method of enrichment. The abattoir samples chosen for investigation were, therefore, mainly those specimens which were positive on one but not on all three solid selective media. There were, however, certain abattoir samples which were *apparently negative on all three selective agars* but which we wished to examine in some detail for particular epidemiological reasons. The abattoir swabs in Table 1 therefore were not consecutive specimens and were chosen somewhat arbitrarily.

The river water specimens were approximately 100 ml. in volume and 10 × 10 ml. were examined at a time. They were diluted with 100 ml. double strength selenite F broth and five of the inoculated broths were incubated at 37° C. and 5 at 43° C. Previous work had shown that it was advisable to use both temperatures in the examination of sewage-polluted river water (unpublished). After incubation, the enrichment broths were subcultured only to de Loureiro's modification of Wilson & Blair's medium. The plates were incubated at 37° C. for 48 hr. before being read. Any apparently negative Wilson & Blair plates were then examined by secondary enrichment. In this series (Table 2) the samples were consecutive and unselected.

METHODS

Each apparently negative selective agar was rubbed over with an ordinary throat swab to remove the surface growth. This growth was suspended in 0·2 ml. of saline in a bijou bottle. A sterile Pasteur pipette with a bulb at the distal end of the capillary stem (Fig. 1) was then partially filled, by suction with a rubber teat, with 0·2 % nutrient agar. The upper surface of soft agar, at this stage, was allowed to come up to the level of the neck of the pipette barrel. The bacterial suspension was next sucked into the bulb at the lower end of the stem underneath the soft agar and in contact with it. A small air space, introduced by suction below the suspension, enabled the lower end of the stem to be sealed in a flame. The bulbous portion of the capillary was necessary to accommodate the 0·2ml. of dense bacterial suspension, otherwise the pipette and its manipulation was identical with that originally described by Harvey & Price (1961). The charged pipette was placed upright in a test tube with a plug of cotton-wool on the bottom and the whole

incubated at 37° C. for 24 hr. During incubation the growth at the lower end of the pipette spread up the stem and appeared on the surface of the agar. This surface growth was subcultured to deoxycholate citrate agar, brilliant-green MacConkey agar and de Loureiro's modification of Wilson & Blair's medium for the abattoir samples. In the series of river water specimens only brilliant-green MacConkey and de Loureiro's medium were used for plating. The selective agars were incubated at 37° C. for 24–48 hr. The brilliant-green plates were examined at 24 hr. and the deoxycholate citrate agar and de Loureiro plates at 48 hr. The 48 hr. incubation of *both* these plates was particularly important. Suspicious colonies were picked and examined in the usual manner.

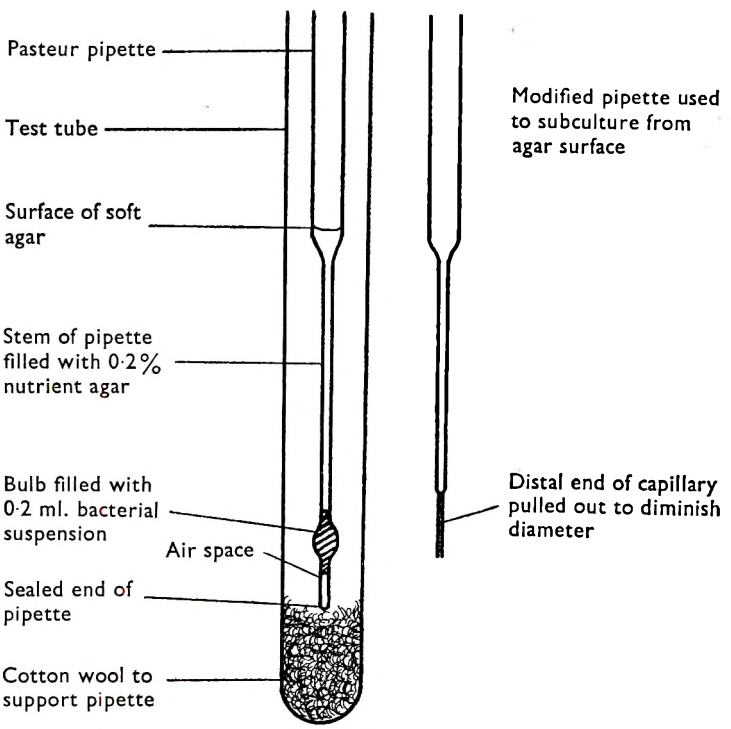


Fig. 1

RESULTS

The results are given in Tables 1, 2 and 3. It is obvious that apparently negative selective agars can often be converted to positive by this technique, and it is particularly noteworthy that six abattoir samples, subcultured from selenite broth in the preliminary examination on all *three* selective media, would have been reported as negative had the secondary enrichment technique not been used.

In order to show that the method was of general application, it was important to observe the range of serotypes which were successfully isolated. These serotypes are listed in Table 3. Both abattoir and river isolations are included. The method was found unsuitable for the isolation of *S. typhi*, *S. dublin* and *S. cholerae-suis*.

It was also unsuccessful in the isolation of the only strain of *S. paratyphi A* encountered in this laboratory during the investigation.

In a few samples the suspensions of growth from the selective agars were plated

Table 1. *Abattoir swabs*

Gross total of swabs positive by all techniques: 44.

Number of swabs which would have been reported as negative had the secondary enrichment technique not been used: 6.

Medium	No. of apparently negative plates examined	No. found positive after passage of growth suspension through pipette
Deoxycholate citrate agar	18	14
Brilliant-green MacConkey	27	19
De Loureiro medium	21	13

Table 2. *River water samples*

	ml.
Volume of sample	100
Total no. of 100 ml. samples examined	190
Total no. of samples positive without secondary enrichment	132
Total no. of samples positive by primary and secondary enrichment combined	158
'Negative' samples converted to positive by secondary enrichment	26

The combination of primary and secondary enrichment gives significantly better results than primary enrichment alone: $\chi^2 = 9{\cdot}8$; $P = 0{\cdot}002$.

Table 3. *Serotypes isolated from abattoir and river samples by pipette technique*

S. agama
S. anatum
S. blockley
S. bovis-morbificans
S. brandenburg
S. bredeney
S. butantan
**S. clifton* (salicin non-fermenting gelatin liquefying)
S. derby
S. eastbourne
S. give
S. heidelburg
S. kingabwa
S. meleagridis
S. oranienburg
S. panama
S. paratyphi B, phage types: 1, Dundee var. 1, 1 var. 1, Battersea
S. paratyphi B var. *odense*
S. poona
S. richmond
S. saint-paul
S. tennessee
S. thompson, phage type 15
S. typhi-murium, phage types: 12*a*, 4, 19, U 165, 1*a*, 32, 9, 12, untypable, 22

* The original description of *S. clifton* was of an organism fermenting salicin (Douglas & Taylor, 1954). The strain isolated in Cardiff failed to ferment 1 per cent salicin peptone water in 21 days.

before passage through the pipettes and these platings were compared with similar subcultures made after passage. The comparisons confirmed that enrichment was taking place.

DISCUSSION

It is unlikely that the 'negative' selective agars could be explained by any lack of efficiency of the plating media. Salmonellas are regularly isolated on all three media from a variety of materials. The brilliant-green MacConkey agar has acted as our standard plating medium for many years (Harvey, 1956) and the routine use of brilliant-green agars in Europe and America for salmonella isolation (Meat Hygiene, 1957) suggests that our own preference for this medium is not misplaced. The de Loureiro plates are regularly checked for their ability to grow a very wide range of salmonella serotypes (Harvey & Price, 1962) and have been found satisfactory for the isolation of *S. typhi* from stools, water supplies (Harvey & Price, 1964), sewage effluent and swabs from a clothes washing machine in a local mental hospital. Our deoxycholate citrate agar gives rich growths of salmonellas and shigellas from stools and when incubated for 48 hours gives almost as good results as brilliant-green MacConkey in the examination of abattoir swabs. It is an essential plating medium for the isolation of *S. dublin*.

Capillary pipettes, although essential to some techniques, for economy of sera (Harvey & Price, 1961; Harvey & Price, 1962), are fragile and are not best suited to routine purposes. It is interesting that a similar observation was made by Carnot & Garnier in 1902. We have, therefore, modified the above technique for the examination of animal feedingstuffs and have incorporated it into our laboratory routine. By means of this modification we have more than doubled our isolations of salmonellas from 25 g. quantities of animal feedingstuffs. The results of this latter investigation will be the subject of a further communication.

SUMMARY

A method of secondary enrichment is described suitable for the isolation of a wide range of salmonella serotypes from abattoir swabs and polluted river water. The technique does not employ selectively toxic chemicals.

REFERENCES

Carnot, P. & Garnier, M. (1902). Sur la technique des cultures en tubes de sable. *C.r. Séanc. Soc. Biol.* **54**, 748.

Carnot, P. & Weill-Hallé, B. (1915). Culture en 'tubes de sable' pour le diagnostic rapide de la fièvre typhoïde et le dépistage des porteurs de germes. *C.r. hebd. Séanc. Acad. Sci., Paris* **160**, 148.

Douglas, S. Hilda & Taylor, Joan (1954). Nine new salmonella species isolated from imported tortoises. *Mon. Bull. Minist. Hlth* **13**, 158.

Friedberger, E. (1919). Eine neue methode (Kapillarsteigmethode) zur Trennung von Typhus und Koli nebst allgemeinen Untersuchungen über das kapillare Steigvermögen der Bakterien im Filtrierpapier. *Münch. med. Wschr.* **66**, 1372.

Friedberger, E. & Putter, E. (1920). Weitere Versuche mit der Kapillarsteigmethode. *Münch. med. Wschr.* **67**, 398.

Harvey, R. W. S. (1956). Choice of a selective medium for the routine isolation of members of the salmonella group. *Mon. Bull. Minist. Hlth* **15**, 118.

HARVEY, R. W. S. (1957). The epidemiological significance of sewage bacteriology. *Br. J. clin. Pract.* **11**, 751.

HARVEY, R. W. S. & PHILLIPS, W. P. (1955). Survival of *Salmonella paratyphi B* in sewers: its significance in investigation of paratyphoid outbreaks. *Lancet* ii, 137.

HARVEY, R. W. S. & PHILLIPS, W. P. (1961). An environmental survey of bakehouses and abattoirs for salmonellae. *J. Hyg., Camb.* **59**, 93.

HARVEY, R. W. S. & PRICE, T. H. (1961). An economical and rapid method for H antigen phase change in the salmonella group. *Mon. Bull. Minist. Hlth* **20**, 11.

HARVEY, R. W. S. & PRICE, T. H. (1962). Salmonella serotypes and arizona paracolons isolated from Indian crushed bone. *Mon. Bull. Minist. Hlth*, **21**, 54.

HARVEY, R. W. S. & PRICE, T. H. (1964). The isolation of *Salmonella typhi* from selenite enrichment media. *Mon. Bull. Minist. Hlth* **23**, 233.

HARVEY, R. W. S. & THOMSON, S. (1953). Optimum temperature of incubation for isolation of salmonellae. *Mon. Bull. Minist. Hlth* **12**, 149.

INO, J. & GRABER, C. D. (1955). Recovery of salmonella from contaminated cultures. *U.S. arm. Forces med. J.* **6**, 586.

LOUREIRO, J. A. DE (1942). A modification of Wilson & Blair's bismuth sulphite agar (stabilized stock solutions). *J. Hyg., Camb.* **42**, 224.

MEAT HYGIENE (1957). *Monograph Ser. W.H.O.* no. 33, p. 405.

PIJPER, A. (1952). Dimensional differentiation, filtration and separation of bacteria. *J. Path. Bact.* **64**, 529.

J. Hyg., Camb. (1966), **64**, 367
Printed in Great Britain

Enumeration of *Clostridium welchii* in the faeces of varying sections of the human population

By ROSS G. A. SUTTON*
Commonwealth Health Laboratory, Lismore, N.S.W., Australia

(*Received* 1 *March* 1966)

INTRODUCTION

The work of Hobbs *et al.* (1953) showing that heat-resistant, non-haemolytic *Clostridium welchii* was a cause of food poisoning began a series of investigations to determine the incidence of this organism in varying sections of the population (Leeming, Pryce & Meynell, 1961; Collee, Knowlden & Hobbs, 1961).

Most of such investigations centred on the qualitative distribution of the organism, and little quantitative work has been carried out on the viable cell counts of *Cl. welchii* in faeces. Smith & Crabb (1961), while investigating the development of faecal flora of animals, carried out counts on a small group (ten persons) of human faeces. They obtained a median count of 1585 *Cl. welchii* per gram. Collee *et al.* (1961) and Goudie & Duncan (1956) both used semi-quantitative techniques and recorded large numbers of *Cl. welchii* present on many occasions. No actual values were given. In the light of these results it was decided that a series of quantitative counts of *Cl. welchii*, both haemolytic and non-haemolytic, would be of some value.

The investigation was intended to compare the counts obtained in groups of healthy individuals, in symptomless excretors of heat-resistant *Cl. welchii* and in a small group of persons associated with an outbreak of food poisoning due to *Cl. welchii*. It was also hoped to determine the value of viable cell counts as a diagnostic tool in investigating outbreaks of food poisoning attributed to *Cl. welchii*.

MATERIALS AND METHODS

Media used

The egg yolk medium was that of Willis & Hobbs (1958) with the medium base adjusted to pH 7·0. Other media used were blood agar (7·5% defibrinated horse blood added to oxoid blood agar base) and Robertson's cooked meat medium.

Neomycin sulphate (Upjohn) was added to the above media, excepting the cooked meat medium, at a concentration of 100 μg./ml. At this concentration the growth of most other organisms found in faeces was suppressed, while the growth of *Cl. welchii* was unaffected. Increasing the concentration to 250 μg./ml. reduced the count obtained with some strains of *Cl. welchii* by 50%.

* Present address: Food Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W.9.

Sources of faecal specimens

Two hundred and fifty faecal specimens were collected in sterile glass containers from three distinct classes of the population. These were: class A, primary school children; class B, members of the general population attending their own general practitioner for miscellaneous minor illnesses; class C, aboriginal persons. In addition faecal specimens were collected from a small group of persons associated with an outbreak of food poisoning due to *Cl. welchii*.

Method of examination

All faecal specimens were examined for *Cl. welchii* by the following three techniques.

1. *Total viable cell counts* of *Cl. welchii* in faeces were carried out by a modified Miles and Misra technique of Smith & Crabb (1961). One gram of faeces was inoculated into 9 ml. of Ringer's solution and four to six glass beads (2 mm. diameter) were added. The mixture was shaken on a mechanical shaker at low speeds for 5 min. This was found to be sufficient to emulsify the faeces. It was then filtered through sterile muslin, and used as a 1/10 dilution of faeces. From this, 1/100 and 1/1000 dilutions were prepared and counts were made using the technique of Miles & Misra (1938). The plates were incubated anaerobically for 24 hr. at 37° C.

2. *Heat-resistant spore counts* were carried out on the filtered 1/10 faecal suspension used above. After doing the total cell counts the 1/10 suspension was held at 100° C. for 30 min., then allowed to cool. The counts were carried out by inoculating 5 drops from a 50 drop per ml. pipette on one plate, and in addition by flooding a second plate with 1 ml. of the boiled suspension. On this latter plate spores could be detected in numbers as low as 10/g. of faeces. The experimental error associated with such low counts would of course be high.

3. *Enrichment technique for the detection of heat resistant spores of* Cl. welchii. This was a modified method of Hobbs *et al.* (1953) One to two grams of faeces were inoculated into tubes of cooked meat medium which were then held at 100° C. for 60 min. and incubated overnight at 37° C. In this way any heat-resistant spores present were concentrated. The tubes were then subcultured on to the egg yolk and blood agar media and incubated anaerobically for 24 hr. Results were recorded as presence or absence of heat resistant spores of *Cl. welchii*.

In order to test the reliability of the Miles & Misra method with faecal suspensions, a known inoculum of *Cl. welchii* was added to a series of tubes containing 9 ml. of Ringer's solution with and without the addition of 1 g. of sterilized faeces, and counts were carried out. The counts in the presence of faeces were slightly lower than those without faeces, but they were quite reproducible.

RESULTS

It was found impossible to make accurate separate counts of haemolytic and non-haemolytic colonies, as faecal matter, particularly in the 1/10 dilution,

obscured observation of haemolysis. Values given in the quantitative section (Table 2) therefore represent total counts.

Of the 250 specimens examined *Cl. welchii* was identified in 175. In the majority of cases only haemolytic *Cl. welchii* was detected; non-haemolytic *Cl. welchii* were isolated from only thirty-nine of the specimens. Twenty-two of the specimens showing non-haemolytic colonies also yielded heat-resistant spores by the enrichment technique. The additional seventeen non-haemolytic strains, isolated from specimens that did not produce spores by the enrichment technique, were further examined. The spore cultures were obtained by the methods described by Hobbs *et al.* (1953). These spores failed to survive 100° C. for 30 min. in cooked meat medium.

Table 1. *Distribution of haemolytic and non-haemolytic* Clostridium welchii *in selected classes of the population*

Class	Number examined	Number showing haemolytic	Number showing non-haemolytic	Heat resistant spores
A	100	69	8	1
B	50	35	5	3
C	100	71	26	18
Total	250	175	39	22

Table 2. *Primary data: viable counts of* Clostridium welchii *in faeces of selected classes of the population; counts are expressed as colonies per drop* (0·02 *ml.*) *of a* 1/100 *faecal suspension*

Range Colonies/drop (0·02 ml.)	Number of specimens within range			
	Class A	Class B	Class C	Overall
Less than 1	33	16	30	79
1– 10	21	10	24	55
11– 20	16	8	14	38
21– 40	12	5	11	28
41– 60	7	4	9	20
61–100	7	4	6	17
101–200	1	1	3	5
201–300	2	2	2	6
350	1	—	1	2

The results for the individual classes are given in Table 1.

By quantitative methods it was possible to record *Cl. welchii* in as small a number as 100/g. of faeces, but because of sampling error such low counts would of course be unreliable. Table 2 gives the primary data for the counts obtained with the varying classes. It can be seen that over 50 % of the specimens in all classes were within the range $0–5{\cdot}0 \times 10^4$ organisms per gram. (i.e. less than ten colonies per drop as shown in Table 2), while 95 % of the specimens contained 5×10^5 or less organisms per gram.

From the primary data given in Table 2, the mean, standard deviation and median count were calculated. These values are given in Table 3.

Inspection of these results immediately shows that the counts are scattered widely about the mean, with a very large standard deviation. They obviously do not represent a normal frequency distribution curve. In such a series of results one or two extreme readings will unduly influence the mean count, so it was considered that the median was the more informative way of expressing these results.

The variation in the daily excretio n of *Cl. welchii* was investigated by examining six specimens—2 per week for 3 weeks—from the same person using the same techniques. The results ranged from $1{\cdot}5 \times 10^3$ to $2{\cdot}05 \times 10^5$ organisms per gram. This is a very significant variation, and might help to explain the large standard deviation and wide range of results given in Tables 2 and 3.

Table 3. *Viable counts of* Clostridium welchii *present in faeces of* (*a*) *selected classes of the population, and* (*b*) *persons associated with an outbreak of* Cl. welchii *food poisoning. Results are expressed in organisms per gram and were calculated from the primary data given in Tables* 2 *and* 4

Class	Median	Mean	Standard deviation
(*a*) A	$3{\cdot}5 \times 10^4$	$1{\cdot}30 \times 10^5$	$2{\cdot}65 \times 10^5$
B	$4{\cdot}5 \times 10^4$	$1{\cdot}49 \times 10^5$	$2{\cdot}72 \times 10^5$
C	$4{\cdot}0 \times 10^4$	$1{\cdot}44 \times 10^5$	$2{\cdot}78 \times 10^5$
Overall	$3{\cdot}7 \times 10^4$	$1{\cdot}40 \times 10^5$	$2{\cdot}71 \times 10^5$
(*b*) Persons associated with food poisoning	$1{\cdot}025 \times 10^7$	$1{\cdot}01 \times 10^7$	$2{\cdot}11 \times 10^6$

Viable cell counts were also carried out after heating the 1/10 faecal suspension to 100° C. for 30 min. (method 2). Although heat-resistant spores could be isolated by enrichment techniques from twenty-two of the specimens examined, only two of these contained heat-resistant spores of *Cl. welchii* in numbers sufficiently high to enable them to be detected by direct methods. The spore counts in these cases were 200 and 500 viable spores per gram.

An actual outbreak of Clostridium welchii *food poisoning*

During the course of these studies, only one small outbreak of food poisoning attributed to *Cl. welchii* was investigated.

Of twelve persons attending a small gathering, seven developed diarrhoea within 12 hr. This was mild, with no vomiting, slight nausea and persisted for only 24 hr. Heat-resistant *Cl. welchii* were present in ten of the twelve persons concerned, and in all seven who developed symptoms of food poisoning.

Total *Cl. welchii* counts were carried out on all ten specimens that contained heat-resistant *Cl. welchii*. The results are given in Tables 3 and 4. These figures differ from those of the normal population in two striking ways.

1. The values are all much higher than those of the normal population.

2. The results are distributed evenly about the mean value; the median and mean being very nearly the same. This therefore represents a special group of persons, and the deviation from the normal viable cell count is significant

(t = 12·5, D.F. = 259, P = less than 0·01). The viable count has increased at least 200-fold (comparing median and not mean counts).

Almost all the organisms present in the above cultures were of the non-haemolytic type—the haemolytic *Cl. welchii* appeared to be present in numbers similar to those found in the general population. It is therefore likely that almost all the *Cl. welchii* present were the heat-resistant type, introduced with the infected meat dish.

Table 4. *Spore and vegetative cell counts of* Clostridium welchii *isolated from faeces during an outbreak of* Cl. welchii *food poisoning*

Viable count per gram of faeces		
Vegetative cells	Spores	Ratio
$5{\cdot}0 \times 10^6$	$1{\cdot}5 \times 10^3$	1/3,333
$6{\cdot}0 \times 10^6$	$3{\cdot}0 \times 10^3$	1/2,000
$7{\cdot}5 \times 10^6$	—	> 1/60,000
$8{\cdot}0 \times 10^6$	$2{\cdot}0 \times 10^3$	1/4,000
$1{\cdot}0 \times 10^7$	$5{\cdot}0 \times 10^2$	1/20,000
$1{\cdot}05 \times 10^7$	$1{\cdot}0 \times 10^3$	1/10,500
$1{\cdot}05 \times 10^7$	$2{\cdot}5 \times 10^3$	1/4,200
$1{\cdot}25 \times 10^7$	$5{\cdot}0 \times 10^2$	1/25,000
$1{\cdot}30 \times 10^7$	$3{\cdot}0 \times 10^3$	1/4,300
$1{\cdot}8 \times 10^7$	$5{\cdot}0 \times 10^3$	1/3,600

Counts were also carried out on the two specimens that did not contain heat-resistant spores. The results were $2{\cdot}1 \times 10^6$ and $3{\cdot}6 \times 10^4$ *Cl. welchii* per gram.

Direct counts, performed on the boiled 1/10 faecal suspension, were able to detect heat-resistant spores in nine of the ten specimens. The spore vegetative cell ratios in these cases were 1:2000 to greater than 1:60000. In the carriers discussed earlier, however, spores were only able to be directly demonstrated in two of the twenty-two specimens examined.

These results are in agreement with the assumption, stated above, that most of the organisms present during active infection are of the non-haemolytic, heat-resistant type. If this had not been the case, there is little likelihood that nine of ten specimens would contain heat-resistant spores in numbers large enough to permit isolation by direct culture methods.

An antiserum prepared from one organism agglutinated all ten organisms isolated from the patients. It also agglutinated a strain of *Cl. welchii* isolated from the incriminated meat dish—a beef caserole—which was eaten by all persons concerned. Serologically this organism did not belong to any of the Hobbs types 1–13 at present considered capable of causing food poisoning.

DISCUSSION

The enumeration of *Cl. welchii* from faeces appears to be possible by the method described. Although there is some reduction in count, the counts are reproducible. Smith & Crabb (1961) used this method to examine the faecal flora of man and animals. They performed numerous daily counts and concluded that as a whole the results were reliable and consistent. Smith (1959) demonstrated by experiments

similar to those described here that the counts on faeces were reproducible. He used a swab and what he terms 'A standardized technique' and one would not expect the results to be as reliable as those using a calibrated dropping pipette.

It was shown that the viable cell count on the same patient varied from week to week. It seems reasonable to expect that the quality and quantity of food and drink, environmental conditions, general health and other factors would have a profound effect on the growth of *Cl. welchii* in the gut, and that under favourable conditions a rapid increase in numbers could occur in a short time, corresponding to the logarithmic phase of growth of organisms in culture. Smith & Crabb (1961) while working with animals noticed that a change in diet produced a change in the bacterial flora of the gut, and that in infants the number of *Cl. welchii* excreted occasionally undergoes a sudden increase in numbers. If the average be taken over a long period of time, however, they point out that the results are consistent. Goudie & Duncan (1956) claim that there is little difference in the day-to-day excretion of *Cl. welchii* provided the consistency of the stool does not change.

When carrying out viable counts from faeces, many variables exist that cannot satisfactorily be eliminated. It is, however, important to know that they do exist. No single figure obtained for a viable count on faeces can be expected to indicate the exact number of organisms being excreted. The method described does, however, offer a reproducible method whereby the bacterial counts of series of specimens can be compared.

Analysis of the results of the quantitative studies shows that the variations between all three classes examined do not represent significant differences. At the beginning of this work it was expected that the aboriginals, having a much higher incidence of heat-resistant *Cl. welchii* (Sutton, 1966) would contain a large group with a much higher viable count. This was not demonstrated, the distribution for all classes being somewhat similar (Table 2). From this, two assumptions can be drawn.

(*a*) The heat-resistant, non-haemolytic *Cl. welchii* are present in much smaller numbers than the haemolytic strains.

(*b*) The growth of the non-haemolytic strain almost completely suppresses the growth of the haemolytic strain, and so is present as a replacement for, and not in addition to the haemolytic strain. This is unlikely as all plates showed many more haemolytic than non-haemolytic colonies. Smith & Crabb (1961) concluded that other organisms generally have little antagonistic effect on the growth of flora normally found in the intestine, and so it would be unlikely that one strain of *Cl. welchii* would completely suppress the other. It is, therefore, more likely that (*a*) above is the correct interpretation of the results. The failure to demonstrate heat-resistant spores readily in the faeces of carriers by direct methods, adds weight to this assumption.

Counts carried out on persons associated with an outbreak of *Cl. welchii* food poisoning show that there is a significant increase in the number of *Cl. welchii* in the faeces of such persons. This could be due to the actual infection leading to multiplication within the host or simply to the diarrhoea associated with the food poisoning leading to an increased output of organisms, even though there is no

actual increase within the intestine. Goudie & Duncan (1956) were able to demonstrate (semi-quantitatively) that *Cl. welchii* could be isolated more often, and in larger numbers, from persons with diarrhoea or after purging. In the results of this work, however, not only was there an increased viable count but heat-resistant spores could be readily isolated by direct methods. It would therefore appear that the increased counts were due to the presence of large numbers of heat-resistant *Cl. welchii*. As this organism was associated with the food poisoning, and is not present in large numbers in the normal population, it is probable that the increased count was due to an actual increase in the organisms present in the intestine, and not simply due to diarrhoea increasing the output of organisms. This predominance of heat resistant *Cl. welchii* in outbreaks must be due to the survival of spores in cooked fresh meats in 'niches', providing favourable conditions for anaerobic growth during long slow cooling

Although the viable counts as a whole have given some useful information, it has been shown that individual counts are subject to too many variables to be of use in laboratory diagnosis. The finding of heat-resistant spores during infection, however, offers what could be a useful tool in investigating an outbreak of food poisoning. Heat-resistant spores could be isolated directly from only two of twenty-two carriers, but from nine of ten persons associated with the outbreak of food poisoning. This is a significant difference ($t = 7{\cdot}16$). Finding spores by direct culture after 30 min. at 100° C. would therefore appear to be evidence for infection due to heat-resistant *Cl. welchii*. This was the case in the single outbreak described here, and as the heat-resistance of spores varies so greatly and different outbreaks have varied so greatly with regard to the number of persons at risk who were actually infected, more figures are needed before this can be adopted as a useful aid in investigating outbreaks of food poisoning due to heat-resistant *Cl. welchii*.

SUMMARY

A method of doing viable counts of *Cl. welchii* has been described that gives reproducible results from faeces. By this method counts were carried out on the faeces of persons in the general population, and those associated with an outbreak of food poisoning due to *Cl. welchii*. There was a significant increase in viable count in those with symptoms of food poisoning. Owing to many variables, single viable counts do not appear to be useful in the laboratory diagnosis of food poisoning, but the detection of heat-resistant spores, by direct culture after boiling, may be of some use. More work must be carried out to substantiate this point.

I wish to acknowledge the helpful advice given by Drs M. A. Bundock and Betty C. Hobbs, and the Director General of Health for permission to publish this work.

REFERENCES

COLLEE, J. G., KNOWLDEN, J. A. & HOBBS, B. C. (1961). Studies on the growth, sporulation and carriage of *Clostridium welchii* with special reference to food poisoning strains. *J. appl. Bact.* **24**, 326.

GOUDIE, J. G. & DUNCAN, I. B. R. (1956). *Clostridium welchii* and neutralizing substances for *Clostridium welchii* alpha toxin in faeces. *J. Path. Bact.* **72**, 381.

HOBBS, B. C., SMITH, M. E., OAKLEY, C. L., WARRACK, G. H. & CRUICKSHANK, J. C. (1953). *Clostridium welchii* food poisoning. *J. Hyg., Camb.* **51**, 75.

LEEMING, R. L., PRYCE, J. D. & MEYNELL, M. J. (1961). *Clostridium welchii* and food posioning. *Br. med. J.* i, 501.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

SMITH, H. W. (1959). The effect of the continuous administration of diets containing tetracyclines and penicillin on the number of drug-resistant and drug-sensitive *Clostridium welchii* in the faeces of pigs and chickens. *J. Path. Bact.* **77**, 79.

SMITH, H. W. & CRABB, W. E. (1961). The faecal bacterial flora of animals and man: its development in the young. *J. Path. Bact.* **82**, 53.

SUTTON, R. G. A. (1966). The distribution of heat-resistant *Clostridium welchii* in a rural area of Australia. *J. Hyg., Camb.* **64**, 65.

WILLIS, A. T. & HOBBS, G. (1958). A medium for the identification of clostridia producing opalescence in egg-yolk emulsions. *J. Path. Bact.* **75**, 299.

J. Hyg., Camb. (1966), **64**, 375
With 2 plates
Printed in Great Britain

The ecology and epidemiology of the pig-bel syndrome in man in New Guinea

BY T. G. C. MURRELL*
Department of Public Health, Kundiawa, New Guinea

J. R. EGERTON† AND ANITA RAMPLING
Department of Agriculture, Stock and Fisheries, Konedobu, Papua

JANET SAMELS AND P. D. WALKER
Wellcome Research Laboratories, Beckenham, Kent, United Kingdom

(*Received* 15 *March* 1966)

Spontaneous gangrene of small intestine, without obvious vascular or mechanical cause, is an extremely rare disease. Bowel gangrene, as such, is rarely associated with the known enteric and dysenteric infections of the bowel. A condition with the pathology of a gangrenous enteritis was first recognized in epidemic numbers in subjects undergoing laparotomy for an 'acute abdomen' at Goroka, New Guinea, early in 1961. The disease was described by the non-specific name of 'necrotizing jejunitis' because initial bacteriological investigations failed to establish a cause (Murrell & Roth, 1963). Beta toxin producing strains of *Clostridium welchii* were subsequently recovered from the bowel contents and faeces of subjects with the condition (Egerton & Walker, 1964). They are considered to be important in the pathogenesis of the disease. 'Pig-bel' has been the name given to the disease because of its aetiological association with the pig-feasting customs of the New Guinea Highlander (Murrell *et al.* 1966).

A similar disease, 'Darmbrand', appeared in epidemic form in north-west Germany in the latter and post-war years, and was believed to be due to *Clostridium welchii* type F (Zeissler & Rassfeld-Sternberg, 1949; Oakley, 1949; Marcuse & König, 1950). A food-borne origin for the disease was established (Hain, 1949), but other workers believed that additional dietary, nutritional and possibly viral factors were associated with the pathogenesis (Pietzonka & Rassfeld-Sternberg, 1950; Hormann, 1948; Siegmund, 1948; Kloos & Brummund, 1951). As a result of the recovery of type C strains from man in New Guinea, the strains of *Cl. welchii* type F, believed to cause 'Darmbrand', have been reclassified on toxicological grounds as a type C variety (Sterne & Warrack, 1964).

Heat resistant type A strains are the only other recognized *Cl. welchii* enteric pathogens of man. These cause food poisoning usually via a medium of re-heated meat dishes (Hobbs *et al.* 1953). These strains produce alpha toxin as the major antigen and are one of the organisms responsible for the ordinary gas gangrene of

* Present address: Department of Medicine, University of Adelaide, South Australia.
† Present address: McMaster Laboratories, C.S.I.R.O., Glebe, New South Wales.

wounds. Strains from other groups, producing different toxins, cause enterotoxaemic diseases in animals, each type having a limited host range. Thus type B is associated chiefly with lamb dysentery (Dalling, 1928), type C with enterotoxaemias of sheep, calves and piglets (McEwen, 1930; Griner & Bracken, 1953; Field & Gibson, 1955), type D with pulpy kidney disease of sheep (Bennetts, 1932), and type E is occasionally found as a saprophyte in the intestines of calves (Bosworth, 1940–43).

This paper describes the epidemiological features of pig-bel and its ramifications with pork consumption in the local population.

MATERIALS AND METHODS

Case records were kept of patients with known and suspected enteritis necroticans during the period January 1961 to November 1964. A total of 210 cases are reviewed in this study, the diagnostic criteria for which have been described previously (Murrell *et al.* 1966; Murrell, 1966).

Bacteriological investigations, except for cases in 1961, were conducted in the Veterinary Laboratory, Port Moresby. Thirty-eight resected specimens of bowel were forwarded packed in ice in a vacuum flask, after tying off a segment proximally and distally. The minimal delay for such specimens in reaching the laboratory was 3 days. Faecal specimens from 107 suspected and contact cases were placed in wide-necked bottles containing buffered glycerol-phosphate transport medium. During 1961, faecal smears were placed in selenite F broth and Robertson's cooked meat media and despatched to the School of Public Health and Tropical Medicine, Sydney, for examination. In the laboratory material from the lumen and necrotic wall were examined for the presence of known aerobic and anaerobic enteric pathogens. Sheep blood agar was inoculated directly from affected bowel surfaces. Material from the lumen (1–2 ml.) was also inoculated into Robertson's cooked meat medium. The latter was placed for 1–2 min. in a boiling water bath and then transferred to the incubator. After 24 hr. subcultures were made on sheep blood agar plates which were incubated anaerobically. Plating on deoxycholate citrate agar plates before and after incubation in enrichment media was used for *Salmonella* and *Shigella* sp. strains. Blood agar plates were incubated aerobically and examined for colonies resembling those of *Bacillus anthracis*. Cultures morphologically and biochemically resembling *Cl. welchii* were forwarded to the Wellcome Research Laboratories for identification by methods described by Egerton & Walker (1964).

Antitoxin titres to the beta toxin of *Cl. welchii* were estimated in 24 proven cases, serial samples being taken from twenty-one persons with the disease. Sera from thirty-eight exposed relatives and from 216 randomly chosen individuals from differing Highland clans were also matched against forty-two control sera taken from Europeans, nineteen in the United Kingdom and twenty-three in New Guinea. The serum was separated, kept under refrigeration and 1 drop of 50% *o*-cresol-ether added to each ml. of serum as a preservative. These were despatched in batches to the Wellcome Research Laboratories for estimation of *Cl. welchii*

beta antitoxin by the method of Glenny, Llewellyn-Jones & Mason, 1931, as modified by Glenny *et al.* 1933.

Epidemiological information was obtained in the field on six different occasions—in June and August 1961 in Upper Asaro; September 1963 in Tambul and Wabag; May and October 1964 in Upper Chimbu; and from June to July 1964 in Chuave, Tari, Lake Kopiago and Baiyer River (Fig. 1). The first three periods coincided

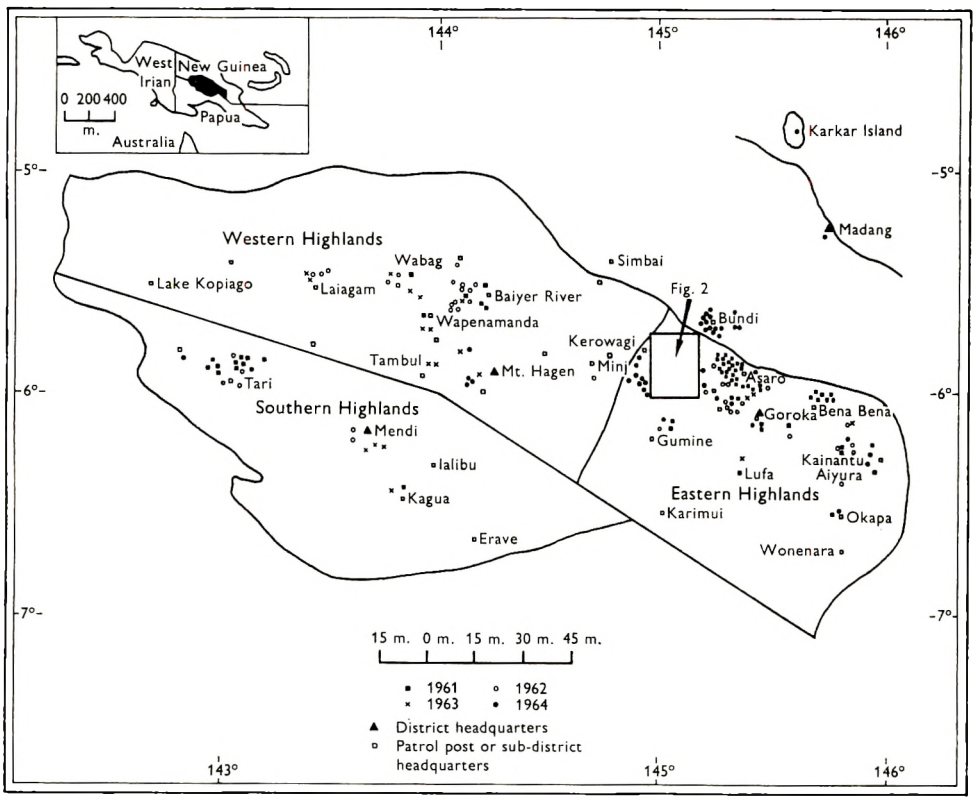


Fig. 1. Geographical distribution of reported cases of pig-bel in the Highlands of New Guinea for the period 1961–1964.

with pig-feasting activities of the local people. Comparative observations of pig-slaughtering methods, preparation of meat for cooking, cooking methods and pork distribution were undertaken. Sources of contamination were looked for: pig carcasses were examined for any noteworthy pathological abnormalities: random samples of faeces and intestinal contents were taken from 322 pigs for bacteriological examination. These were collected aseptically, placed in transport media and sent airfreight to Port Moresby, packed in ice by methods described earlier. Specimens of pig meat in the fresh, cooked and stored state were collected and processed as with other samples. A systematic survey to determine the frequency of *Cl. welchii* type C in the normal population was undertaken during June to July 1964. Clean unsterile waxed paper cups were issued by random sampling to individuals on a basis of a 1 in 5 sample. About 5 g. of faeces were transferred to

wide-necked sterile bottles containing transport medium and forwarded to Port Moresby and from there to London. In the laboratory this was halved to a 1 in 10 population sample. Four hundred and sixty-eight examinations were thus completed. A record of the foods eaten and estimations of the weight and volume of individual meals were made. Temperatures of the cooking process and cooked meat were taken with a thermocouple.

Blood samples were taken from 216 volunteer subjects. These volunteers were representative of seven different Highland groups. These were from Upper Asaro, Chuave and Upper Chimbu (clans in the Eastern Highlands); Baiyer River, Wabag and Lake Kopiago (clans in the Western Highlands), and one clan near Tari in the Southern Highlands.

A population census was conducted in an endemic area at Goromaugo in the Upper Chimbu in October of 1964. An approximate estimate of the disease incidence was obtained by equating the population figures with persons treated for pig-bel in whom the diagnosis was established bacteriologically or serologically.

Information on diarrhoeal disease patterns and causes of death were obtained from records of the Goroka, Tari, Baiyer River and Kundiawa hospitals for periods between 1 January 1962 and 30 November 1964. From the Goroka hospital records a survey of surgical operations undertaken for acute and subacute intra-abdominal conditions for the 4-year period 1 January 1961 to 30 November 1964 was also made.

RESULTS

Disease incidence

The prevalence of pig-bel varied considerably from area to area in the Highlands (Fig. 1). Owing to the lack of adequate demographic records in the Territory, incidence figures were initially assessed from population census figures and established cases. This ranged from 4 per 10,000 in the Wabag district to 22 per 10,000 in the Upper Asaro area of the Eastern Highlands.

A more accurate assessment of the incidence of the disease was made in a census of five clans at Goromaugo in the Upper Chimbu taken in 1964. Following pork feasting in May and August of the same year, seven persons were known to have contracted pig-bel in a population of 1448. The diagnosis was established bacteriologically in three cases, serologically in two and by autopsy in a further two. The incidence in this area was therefore assessed at 48·3 per 10,000. A high prevalence of *Cl. welchii* beta antitoxin in population groups sampled in the Upper Chimbu supported the impression that Goromaugo represented a high incidence area.

Mortality

Two of the seven persons known to have contracted pig-bel during 1964 at Goromaugo died. This gave a mortality of 13·8 per 10,000 population. This compares with the case mortality rate of 36·0%. Excluding the mild forms of the disease this was 49·8%, which was highest in the acute toxic group (84·6%). The mortality rose significantly in the younger and older age groups.

For all ages, enteritis necroticans accounted for 2·1% of deaths at the Goroka and Kundiawa hospitals and 3·5% at the Baiyer River hospital during the period

1 April 1962 and 30 November 1964. These rates were again higher in the 1 to 12 year age group. At Baiyer River 16·1% of deaths occuring in this age group were due to pig-bel.

Outbreaks and geographical distribution

The geographical distribution of cases over the 4 years 1 January 1961 to 30 November 1964 is shown in Fig. 1. Epidemics occurred in the Upper Asaro

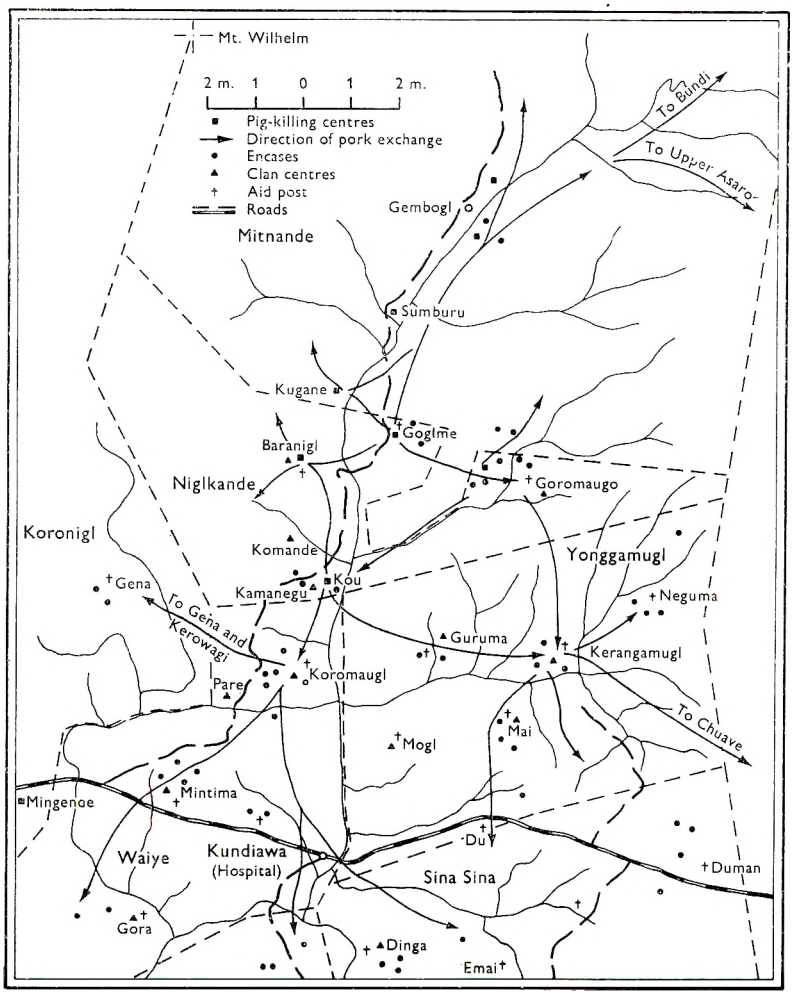


Fig. 2. Local spread of pig-bel in the Upper Chimbu area of the Eastern Highlands. Pig-killing commenced in the Niglkande census division in May of 1964 and concluded in the Sina Sina in October of the same year.

during the months June to September of 1961; in the Tari Basin just prior to this; most of Baiyer River in the Western Highlands in March to June 1962, and in Bundi and the Chimbu throughout 1964.

When pig-feasting activities commenced in May in the Upper Chimbu, pig-bel prevalence rose, as did other diarrhoeal diseases. There was a further increase

following the pig cycles in the Central Chimbu (Fig. 2). The spread of cases is shown along the directional movements of pork shown in Fig. 2. Following the 'Te' festival of the Enga people of the Western Highlands late in 1963, only six cases of the disease were reported. Serological sampling earlier in the year confirmed the impression that the Wabag-Wapenamanda area was a low incidence area. *Cl. welchii* type C was isolated from cases at the Upper Asaro, Chimbu, Baiyer River, Wabag, Wapenamanda and Mendi.

Evidence of local spread

Following pig killing at Goromaugo there was a bridal exchange of pork between members of a Pagakaune line and one man, Ambane Umba, of a Kurumogl line. Ambane subsequently developed a more protracted form of the disease which was confirmed by a rising beta antitoxin and the recovery of *Cl. welchii* type C from his faeces. Sera taken from eight relatives exposed to the same meal and from the man who had prepared the meat all contained significant amounts of beta antitoxin. A further case occurred in a man at Gena (Upper Chimbu) who had eaten pork originating from another Goromaugo clan. Contacts again had immunological evidence of exposure to beta toxin. However, severe infections, confirmed by autopsy or laparotomy, were seen in more than one member of a family at risk on only three occasions.

Owing to difficulties in communication suspected meal remnants could not be obtained.

Seasonal distribution

The data from hospital records indicated that peaks of pig-bel occurred mostly in the 'dry' season between the months of April and September (Fig. 3). Major epidemics coincided with the larger pig-killing ceremonies held at this time of the year because of the prevailing climate and good harvest.

The relationship of pig-bel and epidemics due to other diseases was not investigated in detail. A widespread influenza epidemic preceded the pig killing in the Upper Chimbu in 1964, and a measles epidemic was known to occur from October to December of 1962 in the Western Highlands. Beecroft (1962) in a personal communication noticed that a gastro-enteritis epidemic was prevalent before the pig killing near Baiyer River in April 1962. The seasonal prevalence of all the infective diarrhoeal diseases showed similar trends in the drier months of the year when admission rates at various Highland hospitals were analysed (Fig. 4).

Race, age and sex distribution

Of the 210 persons reviewed in the case series, only one was a European. All other victims, with one exception, were inhabitants of the areas in which they contracted the disease. The exception was a Mount Hagen native working as a plantation labourer on Kar Kar Island, in the Madang District. The European, a 28-year-old Polish linesman, contracted his disease at Tari following a meal of native pork.

Ages and sexes are listed in Table 1. The greatest number of patients were 2–10 years old (52·3%). Children in the 6 to 10-year group made up the largest segment of overall cases (29·0%). The distribution of persons with the disease under 10 years of age was most significant ($P < 0{\cdot}001$) when compared with other decades.

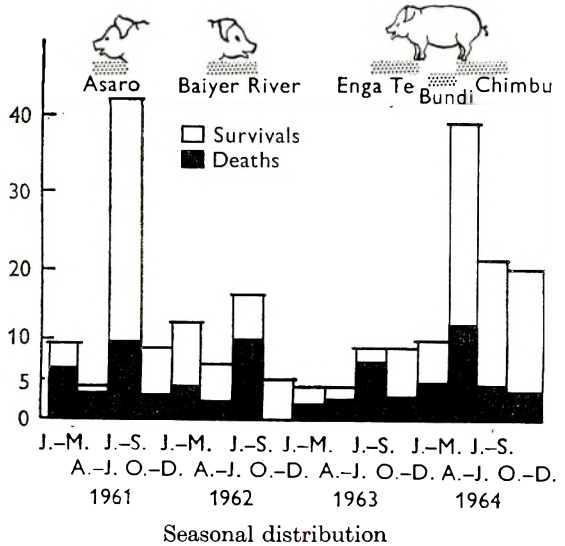


Fig. 3

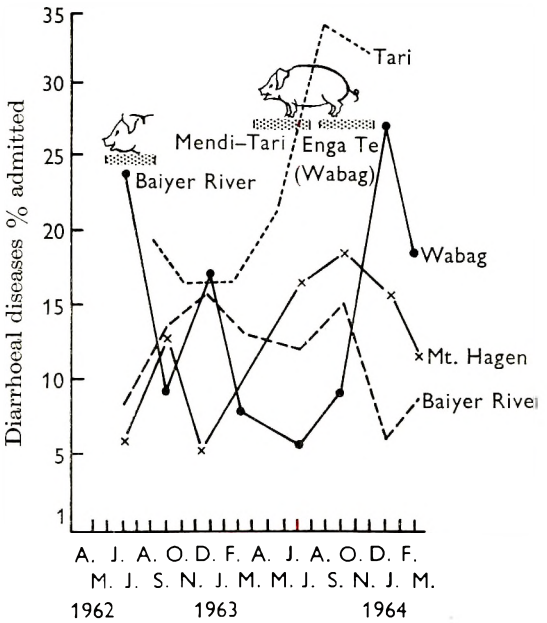


Fig. 4

Fig. 3. Quarterly distribution of pig-bel cases during the period of 1961–64. Pig-feasting activities are indicated at the top of the diagram.

Fig. 4. Prevalence of diarrhoeal disease as measured by percentage of admission for all diseases in four Highland hospitals. Peak rises followed the large pig-kills in the areas served by these hospitals.

Table 1. *Age and sex distribution of* 210 *cases of pig-bel*

Age group	Males	%	Females	%	Total	%
0– 1	1	0·4	2	1·1	3	1·4
2– 5	36	17·1	13	6·2	49	23·3
6–10	46	21·9	15	7·1	61	29·0
11–15	17	8·1	5	2·4	22	10·5
16–20	10	4·8	12	5·7	22	10·5
21–30	13	6·2	8	3·8	21	10·0
31–40	12	5·7	7	3·3	19	9·0
41+	10	4·8	3	1·4	13	6·2
Totals	145	69·0	65	31·0	210	99·9

Males were affected more than females in the ratio of 2·2:1. This ratio was generally maintained in all age groups except in the 16- to 20-year group when more females were recorded with the disease. The numbers recorded in this group, however, were too small to be significant. At Goromaugo, the sex incidence in the

normal population was 5:2 in favour of males. Of the seven persons found to have contracted pig-bel disease in this survey, four were under 10 years of age.

Pig-feasting investigation

The pig is the central figure in all social events—part of the bride price in weddings, currency in compensations, and the main menu dish in feasts celebrating the epochal events in clan and tribal life. It is offered as a sacrifice to placate the spirits of the deceased: it is offered when crops have failed and given medicinally when sickness occurs: wherever, in fact, the ancestor spirits, good or bad, might be. Ownership of pigs, therefore, is not an asset in regard to food supplies. Rather their possession and circulation are values in themselves in enhancing a man's social status and prestige, and form the basis of a barter economy. Within the framework of this credit system large 'pig-kills' occur in cycles when herd numbers have built up sufficiently. These begin within one lineage group and precipitate progressive killings over the ensuing 6 months along well-defined trade routes within clans and tribes (Meggitt, 1958; Brookfield & Brown, 1959). Up to 10,000 pigs may be killed in a 25-mile radius on such occasions.

Observations of a pig-kill

Preparations months in advance took place when long houses were built to accommodate residents and visitors round central courtyard clearings. Tables were also built in readiness to receive the pork sides and quarters for distribution. In the Eastern Highlands 2–4 weeks before a large pig-kill, a smaller celebration took place, the smaller pigs being sacrificed on such an occasion.

All large pig-killing ceremonies occurred at the time of a full moon. Whenever possible they were held during favourable weather which was usually during the dry season. Dancing and singing festivities were held before the killing began. In the Chimbu the pork preparation and distribution took 3–4 days, whereas only 1–2 days was required in the Tari and Wabag areas. A preliminary count of pigs occurred in the 'Te' festival of the Enga tribes (Plate 1*a*). The tethered pigs were clubbed to death by a near relative of the owner, using a 2–3 in. diameter stick. There was no bleeding of the carcass, although some blood from the head wounds spilt out over the ground. Cassowaries, chickens and dogs were occasionally sacrificed along with the pigs but were cooked separately. The hair of the pig was singed over an open fire and the carcasses then lifted on to leaf mats of banana, breadfruit and tree fern leaves. An intricate dissection in the dorsal position was commenced with double lateral incisions in the anterior axillary line (Plate 1*b*). The outermost continued down behind the anus and the inner two met anterior to it. Here, spillage sometimes occurred and the butcher's hands became contaminated directly by pig faeces. The abdominal skin flaps were dissected up towards the head and the thoracic cage opened laterally by axe cuts. A careful butcher then removed the diaphragm, peritoneal sac and contents *in toto*.

The women took the hollow abdominal viscera in their 'bilums' (string carrying bags slung over the head) to the nearest stream for washing. The Chimbu and

Gahuku (Asaro) women everted the small intestine by intussuscepting a stick into the bowel lumen. They siphoned and forced water through the large bowel, the anal skin and anus acting as a funnel. Enga fashion was a little less crude as the whole bowel was split open and cleaned more thoroughly. The bowels were plaited and wrapped in leaves ready for cooking. Stomach was packed with chopped fat, greens and herbs and cooked in the form of a haggis. It was eaten after 'maturing' for 2 or 3 weeks. While the bowel washing progressed, the men continued the final filleting of the carcase, skull, rib cage and backbone being removed in one section. Here, contamination by feet was most apparent as assistants were required to pull the head and spine forward and ventrally.

The butchering completed, which took about 2–4 hr., the night was given to more singing and dancing. Earth pits were dug: long and shallow for the half sides of meat, and deep and wide for the offal and prime cuts. The ovens were lined with banana leaves, tree fern fronds (*Cyathea contaminans*) and leaves of a variety of breadfruit (*Ficus dammaropni*). The meat, sweet potato, taro, corn, bananas and chopped greens were added with pre-heated stones and included in the festive meal. Men handled the long wooden tongs with great dexterity (Plate 2). Layer by layer the oven filled up until the large leaves roof over a final insulating layer formed by the pig's quarters and flanks. Water was poured in and the oven was sealed by another layer of leaves and mounded up with dirt, so that the food was cooked under steam pressure in its own moisture. The juices of all the contents were thus retained in the cooking process. The Chimbu women also cooked bowel and other morsels in wooden barrels.

Table 2. *Cooking time and procedure. The temperature readings were taken from the centre of oven using a thermocoupled pyrometer*

Cooking process	2. x. 63 Time no. 1	Temp. °C	3. x. 63 Time no. 2	Temp. °C
Commenced putting meat in ovens. Water introduced	11.30	38	11.45	35
Sealed with earth and cooking commenced	11.45	82	12.00	79
Temperature rise	11.58	93	12.11	92
Stationary temperature	13.00	104	13.15	102
Started opening pit to remove partially cooked meat (dirt shovelled off)	13.15	104	13.22	104
Exposed meat	13.25	99	13.34	99
Carcasses now cut in half down spinal centre section, meat extracted for disposal, i.e. head, thoracic cavity and everything removed	13.37	93	13.40	83
Actual cooking time	1 hr. 37 min.		1 hr. 34 min.	
Kau-kau and bananas well cooked, soft and crumbly and eaten almost immediately				

Observations near Wapenamanda, indicated in Table 2, show temperature readings taken using a pyrometer. The thermocouple was placed in the centre of the earth ovens and temperatures taken at set time intervals during the cooking. The centre of a hind quarter immediately after removal from an oven after 2 hrs.

cooking had a mean temperature of 78° C. for five pieces sampled. The stones after cooking were a little hotter than warm and could be handled with bare hands.

The results indicated that large chunks of meat were not thoroughly cooked. After cooking the meat rapidly cooled, further handling occurred and the same sources of contamination were present—feet, flies, dogs and so on. It was after cooking that contamination seemed most significant and the arguments and discussions concerning the distribution would continue for a whole day. In transit to its destination, a half side of pork might change hands two to five times and reach consumption point 1–4 days later. A day or more might elapse before the pork was further cut up and a second distribution arranged. Further cooking under similar circumstances increased the chances of contamination and food poisoning. This probably caused the prolonged upper abdominal pain of which so many people complained after eating pork (Nilles, 1950).

From the foregoing, it may be concluded that the likelihood of disease spread was much greater in the large pig ceremonies than in the much smaller marriage and death distributions, where there was a limit to the number of pigs killed and a smaller number of recipients. Bridal payments took place within the framework of the larger ceremonies and stacks of pork quarters and halves were seen in the Upper Chimbu for this purpose.

Sources of infection

Pig and pork

The only notable disease observed in the slain pigs apart from intestinal helminths was intestinal emphysema (Egerton & Murrell, 1965). Between 1 and 4% had macroscopic evidence of enteritis. No cases of anthrax or trichinosis were found. *Cl. welchii* was recovered from the intestinal contents of 53 of 322 pigs chosen at random from pigs killed at Upper Asaro, Tambul and Upper Chimbu. All strains isolated proved to be type A and no type C strains of this organism were recovered. From 115 samples of cooked and uncooked pork, collected mostly from the medial aspect of the hind-quarters where contamination was considered to be most likely, eighteen isolations of *Cl. welchii* type A were obtained. Again no type C strains were recovered. These samples included eighteen cooked bowel specimens. *Escherichia coli* and *Proteus* were also recovered frequently. Remnants of a pig (hair, bone and fat) suspected as a source of origin for five cases of pig-bel, yielded *Cl. welchii* type A but no type C strains. The failure to enlarge the samples from this latter group was due primarily to the late arrival of cases to hospital and the difficulty in obtaining meal remnants. All traces of meat had gone by the time attempts to get such samples were made. The general reluctance of the people to blame pork as a cause also added to this difficulty. Type A strains were recovered from soil samples collected in the Upper Chimbu.

Human population

Antecedent dietary histories were taken from 140 patients with pig-bel: of these, 133 had a prior pork meal and seven persons firmly denied eating pig. The incubation period ranged from 6 hr. to 6 days with a mean of 24 hr. The shorter the

incubation the greater the severity of the disease. Table 3 shows the results of bacteriological examinations of resected bowel and faeces from persons suspected of having pig-bel. *Cl. welchii* was isolated from the lumen and necrotic wall of thirty-six bowel specimens. Seventeen type C strains and nine type A strains were subsequently identified from the isolates. Ten were not submitted for complete identification. The toxicological analysis of eleven beta-producing strains have been reported earlier (Egerton & Walker, 1964). This organism was not recovered from four intestines resected for other causes of strangulation.

Table 3. *Isolation of* Clostridium welchii *in the bacteriological investigation*

Subjects	Total	Negative	Positive	*Cl. welchii* Type A	*Cl. welchii* Type C	*Cl. welchii* Untyped
Surgical resections (bowel smears)	38	2	36	9	17	10
Suspected mild cases and contacts of proven cases (faecal smears)	107	53	54	45	9	0
Follow-up (faecal smears)	17	10	7	6	1	0
Controls						
Bowel resections from other causes	4	1	3	3	0	0
Normal population	468	351	117	117	0	0

Table 4. Clostridium welchii *in normal New Guinea population*

	Faeces examined	Isolation *Cl. welchii* type A	%
Asaro	100	40	40
Upper Chimbu	100	36	36
Wabag	68	12	18
Tari	100	16	16
Lake Kopiago	100	13	13
Total	468	117	25

From a group of 107 in which no surgical intervention was undertaken and which included fourty-four contacts of known cases, *Cl. welchii* strains were isolated from fifty-four faecal specimens (Table 3). Only nine of these proved to be type C strains with the same toxin production as other strains from bowel. *Shigella flexneri* type 2 was isolated from three persons in this group, once in association with *Cl. welchii* type C and twice as the sole demonstrable pathogen.

The 1 in 10 systematic sample of faeces of a normal human population failed to yield any *Cl. welchii* type C strains. This survey was carried out independently of any pig feasting activity, except in the Upper Chimbu where sampling was undertaken 1 month after the pig killing there. The results are shown in Table 4.

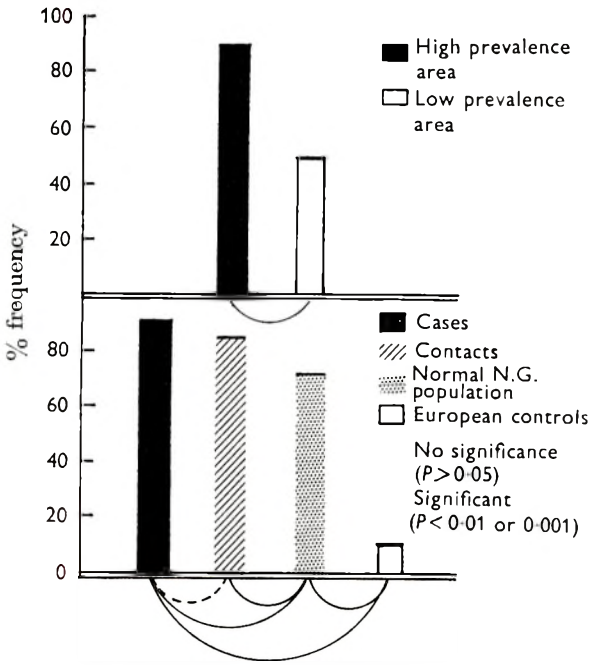
These isolations all proved to be type A strains. No type C strains were recovered from the normal population. The only evidence that type C is of human origin was the recovery of such a strain from a pig-bel patient 12 months after bowel resection (Table 3).

Table 5. *Overall results of beta antitoxin levels*

Group	Place	Units of beta antitoxin per ml. No.	$< \frac{1}{2}$		$\frac{1}{2}$–2	2–5	> 5	$\geqslant \frac{1}{2}$		$\% \geqslant \frac{1}{2}$	
A. Known recent pig feasting											
(i) Low prevalence pig-bel	Wabag	66	34		26	3	3	32		48·5	
(ii) High prevalence pig-bel	Upper Chimbu	39	4		29	5	1	35		89·7	
	Total	105	38		55	8	4	67		63·8	
B. Doubtful recent pig feasting											
(i) Low prevalence pig-bel	Watabung	17	9		8	0	0	8		47·1	
(ii) High prevalence pig-bel	Upper Asaro	20	6		12	1	1	14		70·0	
	Tari	34 } 71	5 } 11		21	7	1	29 } 60		85·3 } 84·5	
	Baiyer River	17	0		13	4	0	17		100·0	
	Total	88	20		54	12	2	68		77·3	
(iii) Unknown prevalence. Recent European contact	Lake Kopiago	23	4		17	2	0	19		82.6	
C. Overall N.G. population (A + B)	Grand Total	216	62		126	22	6	154		71·3	
D. Known cases pig-bel		24	1		9	4	10	23		95·8 } 86·2	
E. Suspected mild cases pig-bel		34	7		20	2	6	27		79·4	
F. Relatives of pig-bel cases		38	6		23	6	3	32		84·2	
G. Follow-up of cases of pig-bel ($> \frac{6}{12}$)		9	4		3	1	1	5		55·6	
H. European controls		42	38		4	0	0	4		9·5	

Serological investigations

Sera were examined for *Cl. welchii* beta antitoxin from seven different population groups (A–H, Table 5). A titre greater than or equal to 0·5 of a unit of antitoxin per ml. was regarded as significant. The last group (H) in Table 5 consisted of a control group of forty-two Europeans. There had been no exposure to native pork except in three of these persons. Four, which included the three at risk, had titres of 0·5–1 unit per ml. In the sera of twenty-four cases of pig-bel, twenty-three had detectable



Statistical significance of differences in % frequency of groups of persons with detectable *Cl. welchii* β antitoxin

Fig. 5. Frequency distribution of *Cl. welchii* antitoxin (*a*) normal populations of high and low pig-bel prevalence and (*b*) in four comparative groups representing cases of pig-bel, their contacts, normal New Guinea population and European controls. Amounts of beta antitoxin 0·5 units per ml. were arbitrarily chosen as significant.

Table 6. *Statistical analysis of antitoxin studies of sera listed in Table* 5

		Comparison with	
	Percentage $\geq \frac{1}{2}$	High prevalence sample	Low prevalence sample
Row (iii) Group B	82·60	$P > 0{\cdot}05$	$0{\cdot}01 > P > 0{\cdot}001$
Group D	95·83	$P > 0{\cdot}05$	$P < 0{\cdot}001$
Group E	79·41	$P > 0{\cdot}05$	$0{\cdot}01 > P > 0{\cdot}01$
Group F	84·21	$P > 0{\cdot}05$	$P < 0{\cdot}001$
Group G	55·56	$P \simeq 0{\cdot}05$	$P > 0{\cdot}05$
Group H	9·52	$P < 0{\cdot}01$	$P < 0{\cdot}001$

antitoxin, ten of which were greater than 5 units per ml. In the overall normal population sample of 216 persons (group C = A + B) 154 (71·3%) had significant antibody levels to the beta toxin. The statistical significance of these results is shown diagrammatically in Fig. 5. In the normal population there were significantly different levels between groups chosen from areas where the disease was

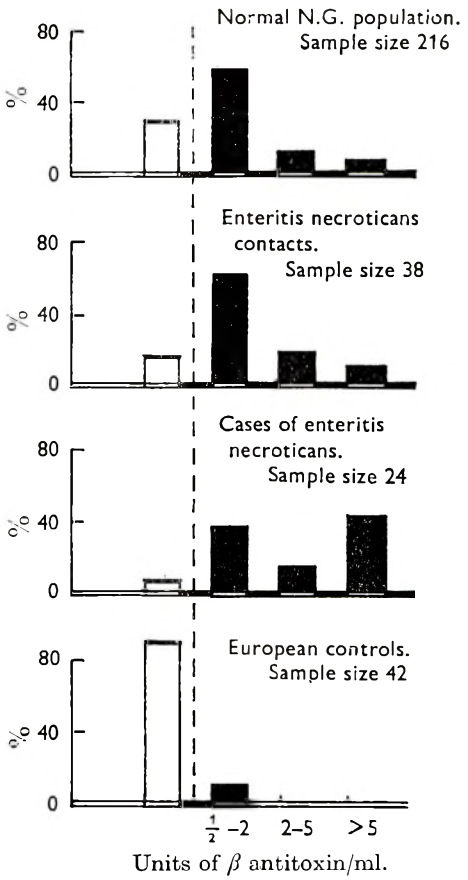


Fig. 6. Quantitative distribution of beta antitoxin in the four groups represented in the lower half of Fig. 8.

thought to have a high and low prevalence respectively, for example, groups A (i) + (ii) and B (i) + (ii). It was not possible to sample a large group of individuals before and after pig feasting. Detectable levels in relatives of persons with known pig-bel were significantly higher than in the normal population, cf. groups C and F (Table 5).

The comparisons of row (iii) of group B representing a 'blind' control group and each of the other groups with the low incidence frequencies and high incidence frequencies of the combined table are shown in Table 6.

Row (iii) group B and groups D, E and F corresponded with high incidence frequencies. Group G corresponded most closely with low incidence frequencies but the percentage $\geqslant \frac{1}{2}$ will be seen to be in a rather equivocal region between the

two extremes. Group H differed significantly from both high and low incidence groups with a percentage of $\geqslant \frac{1}{2}$ of 9·52 which is highly significantly lower than the low incidence percentage.

The number of sera containing more than 2 units/ml. of beta antitoxin was considerably higher in groups D, E and F. This upward trend is shown diagrammatically in Fig. 6. These results suggest that the immune response in individuals in these groups is the result of a greater or more prolonged exposure to beta toxin. They also indicate that antitoxin detection may be diagnostically reliable.

The frequency of detectable beta antitoxin in different age groups is shown in Table 7. It was highest in thirty-one persons above an estimated age of 40 years (90·3%). In the 16- to 20- year age group only 55·6% of those examined had detectable levels. Between these limits the frequencies were evenly distributed between 63·6 and 76·9%. There was no statistical difference in titres detectable in males and females (Table 8).

Table 7. *Age distribution of antitoxin titres in normal population*

Age group	No. examined	Detectable beta antitoxin per ml.	%
0– 5	7	5	71·4
6–10	11	7	63·6
11–15	22	14	63·6
16–20	18	10	55·6
21–30	39	30	76·9
31–40	55	40	72·7
40+	31	28	90·3
Total	183*	134	73·2

* Total population surveyed differs slightly from that in Table 8 because age and sex were not recorded on thirty-three occasions.

Table 8. *Sex distribution of antitoxin titres in the normal New Guinea population*

Sex	No.	Detectable beta antitoxin	%	
M	74	56	75·7	$P > 0{\cdot}05$
F	109	78	71·6	
Total	183	134	73·2	

DISCUSSION

Accurate measurement of the occurrence of enteritis necroticans proved difficult because there was no single pathognomonic criterion of clinical diagnosis. The figure of 48·3 per 10,000 at Goromaugo must serve only as a rough guide to assess the size of the problem.

In North Germany, with the exception of Lübeck, the incidence of Darmbrand ranged from 0·04 per 10,000 in 1946 to 1·77 per 10,000 in 1947. The maximal

incidence was 3·59 in Kiel in 1947 (Kloos & Brummund, 1951). In Lübeck the incidence was much higher, being 16 per 10,000, and the mortality was estimated to be 3·5 per 10,000 (Hansen *et al.* 1949). The mortality figure at Goromaugo was 13·8 per 10,000. The discrepancies between the two German figures were influenced rather by difference in definition and classification of the less severe forms of the disease.

Of 364 Darmbrand cases reviewed by Hansen *et al.* (1949) the overall death rate was 22%. In a series of 355 cases Kloos & Brummund (1951) estimated the case fatality rate at 41·4%. These latter writers were concerned mainly with the severe forms of the disease and included only thirty-eight in the mild group. Three other German authors gave high mortality rates for their case series. Ernst (1948) reported a mortality of 50% in Hamburg, Nissen (1950) a figure of 44% for the Landesteil-Schleswig area, and Griessman (1950) a mortality rate of 46% for 124 cases. These figures compare with an overall New Guinea case fatality rate of 36·0%. This case fatality rate was maximal at 52% in 1963, but fell to 30% in 1964 following the introduction of antiserum therapy in June of that year. The cumulative experience of managing cases preoperatively also helped to bring about this reduction (Murrell & Roth, 1963; Murrell *et al.* 1966).

The German outbreaks of Darmbrand had well defined seasonal distributions over the years 1947–48 and the disease reached pandemic levels during the mid and late summer months (July to September) of those years. The maximum incidence occurred in 1948, and then in the following 2 years declined and the disease disappeared as mysteriously as it had arrived. Jeckeln (1957) who had experience with a large number of patients in Lübeck, expressed the view that this intestinal disease, the most severe he knew, had abated. The seasonal or annual appearance of the disease in New Guinea was generally confined to the middle 6 months of the calendar year. This is in the 'dry' season of the year and as such, gardens were at maximum production and the absence of rain favoured outdoor festivities.

A noteworthy difference in the New Guinea disease from its German counterpart was the age distribution. The number of persons in the 0 to 10 year age groups was significantly higher (53·8%) than in any other decade. This distribution contrasts with Darmbrand, where most cases occurred in the fourth, fifth and sixth decades, although infants under 1 year of age were reported with the condition (Jochims, 1947). The mortality rose in the very young and most elderly patients, which was also the case in New Guinea. The higher incidence in children in New Guinea has similarities with the incidence of enterotoxaemia in sheep, calves, lambs and piglets caused by type D and C strains (Field & Goodwin, 1959). The occurrence of the disease in childhood in New Guinea is readily explained by the dietary practice associated with pork feasting. With the larger pig cycles occurring at 3–7 yearly intervals, it is possible that the younger age groups have an initial exposure to massive pork meals. Infants without teeth are not offered solid foods, and toddlers, who are breast fed up to the age of $2\frac{1}{2}$ to 3 years, are given only token amounts of pork. Only three patients in the series reviewed were under 2 years. The belief that pork imparts strength into the individual is practically demonstrated by encouraging children, especially males, to consume as much

as possible. Particular organs, such as the genitalia, liver and kidneys, are also reserved for children to eat. Burchett (1964—personal communication) reported that in the Baiyer River outbreaks in 1962 children actively refused further meals offered to them by their parents because of their fear of becoming ill.

The reluctance of older people to seek medical care, the general regard that illness in the older generation is due to 'old age' and lower life expectancy, account for the fewer case reports in people over 40 years of age. The life expectancy of the Highlander is probably lower than that estimated in life tables by Scragg (1954) for New Ireland natives of New Guinea.

The sex distribution in New Guinea may be influenced by the greater frequency with which males seek medical care as indicated by a higher bed occupancy rate both in Papua (Campbell & Arthur, 1964) and the Territory as a whole (Department of Public Health, 1964). However, the survey at Goromaugo indicated that twice as many males as females were affected. The women do eat more of the cooked bowel of the pig than the men. If heat resistant spores of the clostridial organisms survive the cooking, as well they might, a higher morbidity of illness should be expected in the females. The New Guinea type C strains did not exhibit the heat resistant quality that the type F strains did in Europe (Egerton & Walker, 1964). More females contracted the disease than males in the 16– to 20-year age group. At marriage feasts the bride and prospective brides are encouraged to consume unusually large amounts of pork to encourage fertility. This possibly explains the distribution of the disease in this group.

The evidence that large scale pig-killing activities influence the prevalence and spread of enteritis necroticans in the Highlands is circumstantial. It would appear from the trends in admission figures that this cultural practice influences the prevalence of all diarrhoeal diseases in the localities of the hospitals reviewed.

The experience of one of us (T. G. C. M.) at Wabag and Kundiawa left the impression that pig-bel varied considerably in density in the two areas. This impression was borne out by the differing titres of beta antitoxin in individuals sampled in the upper Chimbu and Wabag areas after pig feasting. An alternative explanation of the variable prevalence may lie with the actual feasting habits of the tribes concerned. In the Chimbu it became apparent that the admission rate rose as the feasting continued. In the Goroka and Chimbu areas, there was also a small introductory pig-kill which was the overture to the larger massive feast. This took place 2–4 weeks earlier and was not a feature of pig-kills witnessed in the Western Highlands.

A more accurate index of the morbidity and mortality following pig feasting could be obtained by measuring the diarrhoeal attack rate in a population sample along the lines of the WHO team investigations (Ordway, 1960). Difficulties apparent in this approach were found to be a reluctance of the population to implicate pork as a cause of diarrhoea, and the problem of accounting for people before, during and after the feasting, due to the transient migrations which naturally occurred at these times. The proven cases at Goromaugo and the subsequent appearance of documented cases at Kurumogl and Gena after consuming pork prepared by people from Goromaugo supports the argument that

pork is a vector of the disease. A significantly high level of *Cl. welchii* beta antitoxin was found in contact persons at risk. The failure to demonstrate type C strains in other than clinical cases suggests a low incident 'carrier rate' in either the human or porcine population. The prolonged exposure of samples to aerobic conditions before plating probably accounted for this failure because the immunological studies provided strong support for a significant exposure to the beta toxin of *Cl. welchii* in the normal Melanesian population. It is unfortunate that a parallel study was not undertaken in pigs because this may have provided some added information on the origin of *Cl. welchii* type C.

The generally high immune status of the population could explain the absence of massive epidemics of the disease. At Bundi, where many deaths occurred, the previous large pig feast had been held 7 years earlier. Here the population was not as dense as in the Chimbu and a significantly high non-immune population may have built up during the 7 years' absence from massive pork feasting. In this regard, the fluctuations in the disease prevalence simulate those of some other diseases such as chicken pox and measles, their endemicity being dependent on the non-immune pool. In the Chimbu, it was established that the time interval between the larger feasts was only 3 years. This factor could therefore influence the variance of disease from one place to another.

A future investigation of the immune status of new-born infants and toddlers may provide information on the age at which exposure to *Cl. welchii* type C first takes place. Samples taken in the 0- to 5-year age group were too small to make any meaningful conclusions. Examination of faeces for the presence of beta toxin is another important avenue for further elucidation of the epidemiology and aetiology of pig-bel.

In limited experiments with guinea pigs, intraduodenal inoculation of type C strains reproduced the disease whereas feed contamination with this organism failed (Egerton, 1966). Whilst food poisoning seems the most likely cause of pig-bel, this has yet to be demonstrated. Considerable problems face the investigator in determining family outbreaks of food poisoning that may follow pig feasts in New Guinea. The difficulties include the primitive state of the people; the lack of adequate communication, and the deficient pathology services available in Highland hospitals.

The variable incubation period in pig-bel suggests that both exogenous and endogenous infection may be responsible in the pathogenesis. The suggestion of Dische & Elek (1957) that toxic effects of *Cl. welchii* enteritis depend upon the presence of living clostridia in the bowel rather than pre-formed toxin supports this argument.

The pathogenesis probably starts by the action of powerful toxins in the upper intestine and this genesis is then stimulated by additional factors, intrinsic and extrinsic, favouring an overwhelming toxaemic infection of the host. The role that other faecal and oral flora play in this toxaemia requires further investigation. Differential bacteriological analysis of organisms of the bowel flora such as *E. coli*, *Paracolon* sp., *Bacteroides* sp., *Streptococcus faecalis* and *Proteus miribalis* normally present in bowel above obstructed areas (Bishop & Allcock, 1960) needs to be

worked out in the New Guinea disease. It is believed that pig-bel bears an epidemiological relationship to the enterotoxaemias of animals where dietary change and over-feeding play such an important role in their initiation (Bullen & Scarisbrick, 1957). Exogenous infection more probably occurs in the young whilst an endogenous infection by resident gut flora, stimulated by local changes, can be postulated in older age groups. The age distribution of clinical types of pig-bel favours this thesis.

The Highlander eats his pork in hugh quantities over a period of weeks or months during the larger pig festival seasons. Then follows a relative fast until the next celebration. Heavy and continuous pork indulgence therefore occurs only once or at the most twice every 3–7 years or possibly longer. There are occasions, however, when smaller festivities as death, marriage or illness call for a small sacrifice of one or more pigs. The mean weights of pork eaten at such a ceremony were estimated by Venkatachalam (1962) at 29 and 26 g. per day in children in the 10- to 15- and 5- to 10-year age groups respectively. At a large pig-kill at Goglme the mean weight of five pork meals consumed by adults and children was 652 g. Volumetric measurements were not made, but the total engorgement of food at one sitting would approximately amount to between 2 and 3 l., more than half of which was made up of pork. This unaccustomed diet is both qualitatively and quantitatively in excess of the average daily diet.

The diet of the Chimbu people has been studied by several workers (Oomen & Malcom, 1958; Venkatachalam, 1962; Bailey & Whiteman, 1963). This consisted of a daily protein intake of between 25 and 30 g. The calorie intake was between 1850 and 2883, very little of which was made up by fat. The conclusion was that such a low protein intake, mostly arising from vegetable protein, amounted to chronic protein malnutrition, particularly in the very young and elderly. Therefore, an intestinal tract primarily conditioned to a vegetarian diet, the bulk of which is a carbohydrate staple of sweet potato, suddenly becomes confronted with large meals rich in animal protein and fat. The stomach of the Highlander is well conditioned to meals of large bulk, because as much as 1500–2000 g. of sweet potato make up a normal meal (Bailey & Whiteman, 1963). However, in order to handle the qualitative change in dietary content, there must be a change in bacterial flora and enzyme secretion in the gastro-intestinal tract. In children it is possible that this change may induce bowel atony. The high helminthic load, so often present in children of the 4- to 10-year age groups, may adversely influence this change, the nett result being stasis of intestinal content and bowel distension favouring the proliferation of existent and ingested bacteria. Yasnogorodsky (1936) drew attention to the production of paralytic ileus caused by over abundant, unaccustomed, indigestible or spoiled food. In South India, a condition of spontaneous paralytic ileus has also been reported. This is considered to be due to a diet of spoiled millet ragi, the staple food (Roantree, 1949).

Pietzonka & Rassfeld-Sternberg (1950) supported the suggestion of Schütz (1948) that there was a history of an antecedent diet rich in protein, usually of improperly cooked or canned meat preparations, in persons with enteritis necroticans in Germany. Thus in Germany, soon after World War II, a similar situation existed

in the population to that in New Guinea, whereby a population subsisting on meagre rations, was suddenly tempted by an influx of richer foods when hostilities ceased. This unbalanced situation, in which the diet consisted chiefly of carbohydrates, is believed to have been a factor underlying the epidemics in both Norway and Germany.

The success of specific *Cl. welchii* type C antiserum in therapeutic trials (Murrell *et al.* 1966) both supports our hypothesis as to the aetiology of pig-bel and provides an opportunity for the future control of the disease using toxoided preparations. Eradication of the disease and other food borne enteric infections, however, lies in an understanding of the beliefs and customs relating to pig raising and ownership in Highland cultures. The efforts of the social anthropologist, epidemiologist and bacteriologist will have to be married if success is to be achieved in the reduction of the mortality and morbidity which result from unhygienic pork consumption in New Guinea.

SUMMARY

1. Features in the epidemiology of a spontaneous enteric gangrene in the Highlands of New Guinea are described.

2. The disease has been called pig-bel because of its firm association with the pig-feasting practices of the people, which occur in 3–7 year cycles.

3. *Cl. welchii* type C is believed to play an important role in the pathogenesis of the condition. Strains isolated were strongly toxigenic and uniform in their toxin production.

4. A food poisoning aetiology was not proved but circumstantial and immunological evidence suggest that pork may be a vector of the disease.

5. The source of *Cl. welchii* type C was not established.

We wish to acknowledge the early help given by Dr L. C. Rowan of the School of Tropical Medicine, Sydney, and Dr A. White in the early stages of this work. Without the loyal support of hospital and laboratory staffs this work would not have been possible. We are particularly indebted to colleagues, Drs L. Roth, R. Rodrigue, C. Conner and P. Burchett for providing clinical information and material for the study, and to members of the Papua and New Guinea Medical Research Advisory Committee for advice. The work was undertaken with the support of the Director of Public Health, Territory of Papua and New Guinea, and the Director of the Wellcome Research Laboratories.

REFERENCES

BAILEY, K. V. & WHITEMAN, J. (1963). Dietary studies in the Chimbu (New Guinea Highlands). *Trop. geogr. Med.* **15**, 377.

BENNETTS, H. W. (1932). Infectious enterotoxaemia (the so-called braxy-like disease) of sheep in Western Australia. *Bull. Coun. scient. ind. Res., Melb.* 57.

BISHOP, R. E. & ALLCOCK, E. A. (1960). Bacterial flora of the small intestine in acute intestinal obstruction. *Br. med. J.* i, 766.

BOSWORTH, T. J. (1940–43). On a new type of toxin produced by *Cl. welchii*. *J. comp. Path.* **53**, 245.

BROOKFIELD, H. C. & BROWN, P. (1959). Chimbu land and society. *Oceania* **30**, 1.

BULLEN, J. J. & SCARISBRICK, R. (1957). Enterotoxaemia of sheep: experimental reproduction of the disease. *J. Path. Bact.* **73**, 495.

CAMPBELL, C. H. & ARTHUR, A. (1964). A study of 2000 admissions to the medical ward of the Port Moresby General Hospital. *Med. J. Aust.* i, 989.

DALLING, T. (1928). Lamb dysentery. *Vet. Rec.* **8**, 841.

DEPARTMENT OF PUBLIC HEALTH, TERRITORY OF PAPUA AND NEW GUINEA (1962–63). Hospital Disease Statistics.

DISCHE, F. E. & ELEK, S. D. (1957). Experimental food poisoning by *Cl. welchii*. *Lancet* ii, 71.

EGERTON, J. R. & MURRELL, T. G. C. (1965). Intestinal emphysema in pigs in the Western Highlands of New Guinea. *J. comp. Path.* **74**, 35.

EGERTON, J. R. & WALKER, P. D. (1964). The isolation of *Clostridium perfringens* type C from necrotic enteritis of man in Papua–New Guinea. *J. Path. Bact.* **88**, 275.

EGERTON, J. R. (1966). Bacteriological investigation of necrotic enteritis. *Papua New Guin. med. J.* (in the Press).

ERNST, O. (1948). Epidemiologische Beitrag zur Enteritis necroticans. *Dte GesundhWes.* **3**, 262.

FIELD, H. I. & GIBSON, E. A. (1955). Studies on piglet mortality; *Clostridium welchii* infection. *Vet. Rec.* **67**, 31.

FIELD, H. I. & GOODWIN, R. F. W. (1959). The experimental reproduction of enterotoxaemia in piglets. *J. Hyg., Camb.* **57**, 81.

GLENNY, A. T., BARR, M., LLEWELLYN-JONES, M., DALLING, T. & ROSS, H. E. (1933). Multiple toxins produced by some organisms of the *Cl. welchii* group. *J. Path. Bact.* **37**, 53.

GLENNY, A. T., LLEWELLYN-JONES, M. & MASON, J. H. (1931). The intracutaneous method of testing the toxins and antitoxins of the gas gangrene organisms. *J. Path. Bact.* **34**, 201.

GRIESSMAN, H. (1950). Über den Darmbrand *Arch. klin. Chir.* **254**, 1.

GRINER, L. A. & BRACKEN, F. K. (1953). *Cl. perfringens* (type C) in acute haemorrhagic enteritis of calves. *J. Am. vet. med. Ass.* **122**, 99.

HAIN, E. (1949). Origin of *Cl. welchii* type F infection. *Br. med. J.* i, 271.

HANSEN, K., JECKELN, E., JOCHIMS, J., LEZIUS, A., MEYER-BURGDORFF, H. & SCHÜTZ, F. (1949). *Darmbrand—Enteritis Necroticans*. Stuttgart: Georg Thiem, Verlag.

HOBBS, B. C., SMITH, M. E., OAKLEY, C. L., WARRACK, G. H. & CRUICKSHANK, J. C. (1953). *Clostridium welchii* food poisoning. *J. Hyg., Camb.* **51**, 75.

HORMANN, H. (1948). Zur Ätiologie der nekrotisierenden Enteritis. *Klin. Wschr.* **26**, 319.

JECKELN, E. (1957). Fortschritte und Probleme im der Dünndarm pathologie. *Verh. dt. Ges. inn. Med.* **63**, 404.

JOCHIMS, J. (1947). Klinische Beobachtungen über den akuten Darmbrand junger Kinder. *Dtsch. med. Wschr.* **72**, 166.

KLOOS, K. & BRUMMUND, W. (1951). Epidemiologische Studien über die Enteritis gravis in Nordostholstein (ausser Lübeck). *Z. Hyg. InfektKrankh.* **132**, 64.

MCEWEN, A. D. (1930). *B. paludis*: A new species of pathogenic anaerobic bacterium. *J. comp. Path.* **43**, 1.

MARCUSE, K. & KÖNIG, J. (1950). Bakteriologishe Befunde bei Jejunitis nekroticans. *Zentbl. Bakt. ParasitKde.* Abt I, Orig. **156**, 107.

MEGGITT, M. J. (1958). The Enga, of the New Guinea Highlands. Some preliminary observations. *Oceania* **28**, 253.

MURRELL, T. G. C. (1966) *Pig-bel—Enteritis necroticans*. M.D. Thesis, University of Adelaide.

MURRELL, T. G. C. & ROTH, L. 1963). Necrotizing jejunitis: A newly discovered disease in the Highlands of New Guinea. *Med. J. Aust.* i, 61.

MURRELL, T. G. C., ROTH, L., EGERTON, J. R., SAMELS, J. & WALKER, P. D. (1966). Pig-bel—enteritis necroticans. A study in diagnosis and management. *Lancet* i, 217.

NILLES, J. (1950). The Kuman of the Chimbu Region, Central Highlands, New Guinea. *Oceania* **21**, 25.

NISSEN, G. (1950). Cited by Kloos, L. & Brummund, W. (1951). *Z. Hyg. InfektKrankh.* **132**, 64.

OAKLEY, C. L. (1949). The toxins of *Cl. welchii* type F. *Br. med. J.* i, 269.

OOMEN, H. A. P. C. & MALCOLM, S. H. (1958). Nutrition and the Papuan child. *Tech. Pap. S. Pacif. Commn.* 118.

ORDWAY, N. K. (1960). Diarrhoeal disease and its control. *Bull. Wld Hlth Org.* **23**, 73.

PIETZONKA, H. & RASSFELD-STERNBERG, L. (1950). Weitere bakteriologisch gesicherte fälle von Enteritis necroticans in Hamburg und Ditmarschen (März 1948 bis März 1949). *Klin. Wschr.* **28**, 425.

ROANTREE, W. B. (1949). Spontaneous paralytic ileus. *Br. J. Surg.* **36**, 352.

SCHÜTZ, F. (1948). Zur Atiologie der nekrotisierenden Enteritis. *Medsche Klin.* **43**, 341.

SCRAGG, R. F. R. (1954). *Depopulation in New Ireland. A study of demography and fertility.* M.D. Thesis, University of Adelaide.

SIEGMUND, H. (1948). Zur Pathologie und Pathogenese der als Darmbrand bezeichneten Enteritis necroticans. *Klin. Wschr.* **26**, 33.

STERNE, M. & WARRACK, G. H. (1964). The types of *Clostridium perfringens*. *J. Path. Bact.* **88**, 279.

VENKATACHALAM, P. S. (1962). A study of the diet, nutrition and health of the people of the Chimbu area. *Department of Public Health, Territory of Papua and New Guinea, Monograph*, no. **4.**

YASNOGORODSKY, A. Y. (1936). *Vyestn. Khir.* **44**, 67. Abstr. *Zent. Org. Chir.* (1937) **84**, 498.

ZEISSLER, J. & RASSFELD-STERNBERG, L. (1949). Enteritis necroticans due to *Clostridium welchii* type F. *Br. med. J.* i, 267.

EXPLANATION OF PLATES

PLATE 1

(*a*) A preliminary count of pigs at a 'Te' festival near Wabag in the Western Highlands of New Guinea.

(*b*) Dissection and evisceration of singed carcasses in progress. The animals are not bled prior to butchering (Upper Chimbu).

PLATE 2

Half sides of pork are placed in a long leaf lined earth oven and cooking occurs from pre-heated stones. (Enga 'Te' pig-kill at Tambul in the Western Highlands.)

(*a*)

(*b*)

(*Facing p.* 396)

The Journal of
GENERAL MICROBIOLOGY

Vol. 44, No. 2 *45s.* *August 1966*

Subscription £5 per volume of 3 parts. *Orders should be sent to*

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD
LONDON, N.W.1

CONTENTS

The Journal of Hygiene is issued in four parts to a volume.

Quotations can be given for back volumes on application.

Papers for publication should be sent to Dr R. M. Fry, Department of Pathology, Tennis Court Road, Cambridge. Other communications should be addressed to the University Printing House, Cambridge.

Papers forwarded to the Editor for publication are understood to be offered to *The Journal of Hygiene* alone, unless the contrary is stated.

Contributors receive twenty-five copies of their paper free. Additional copies, not exceeding two hundred (except in special cases), may be purchased; these should be ordered when the final proof is returned.

The subscription price is £4. 10*s*. per volume (post free), payable in advance; single numbers 30*s*. net. Subscriptions may be sent to any Bookseller, or to The Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1.

The subscription price in the U.S.A. is $17.50. Single parts $5.00. Enquiries should be addressed to the American Branch of the Press at 32 East 57th Street, New York, N.Y. 10022.

Printed in Great Britain at the University Printing House, Cambridge
(*Brooke Crutchley, University Printer*)