Vol. 68, No. 2 June 1970

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 £7 *net* ($23.00) (4 parts)

Single parts 40*s. net* ($6.50)

The Journal of Hygiene

Papers for publication should be sent to Dr R. M. Fry, Department of Pathology, Tennis Court Road, 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 may be purchased; these should be ordered when the final proof is returned.

SUBSCRIPTIONS

The Journal of Hygiene is published quarterly.

Single parts 40*s*. net ($6.50 in the U.S.A.) plus postage.

Four parts form a volume. The subscription price of a volume (which includes postage) is £7 net ($23.00 in the U.S.A.).

Second class postage paid at New York, N.Y.

Quotations for back volumes may be obtained from the publisher.

Orders should be sent to a bookseller or to the publisher:

Cambridge University Press

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

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

J. Hyg., Camb. (1970), **68**, 159
Printed in Great Britain

In vitro method for safety testing of foot-and-mouth disease vaccines

By E. C. ANDERSON, P. B. CAPSTICK* and G. N. MOWAT
The Animal Virus Research Institute, Pirbright, Surrey
and F. B. LEECH
Rothamsted Experimental Station, Harpenden, Herts.

(*Received* 14 *August* 1969)

SUMMARY

The susceptibility of the tissue culture system to small amounts of residual live virus was not influenced by the inactivated antigen present. The depth of inoculum over the cell sheet did not affect results. Negative cultures frequently gave positive first (but not second or later) sub-cultures.

Baby hamster kidney cells were always more sensitive than cattle tongues to infection with any of the strains used.

Confidence in the safety test depends on the number of vaccination doses used; the tissue culture test can be made much more reliable than the cattle test because it is not limited to the 15 ml. of inoculum that restricts the cattle test.

INTRODUCTION

Henderson (1952*b*), when reporting on tests for non-infectivity of foot-and-mouth disease (FMD) vaccines, concluded that the intradermal cattle tongue inoculation test was then the best available. At that time, vaccines were inactivated in the presence of adjuvants, and tissue culture tests could not be used because of the cytotoxic effects of the adjuvants. Now that the virus can be inactivated in the absence of adjuvant, tissue cultures can be used to test for any virus remaining active in the inactivated suspensions.

Because vaccines prepared from virus grown in the baby hamster kidney (BHK 21, clone 13) cell line (Macpherson & Stoker, 1962) are now widely used, it seemed logical to investigate the value of this cell line for innocuity tests. The work described in this paper was directed first to an examination of the factors likely to affect the sensitivity of the tissue culture test and, second, to a series of comparisons between the cattle tongue test and the BHK tissue culture system.

* Present address: Wellcome Institute for Research on Foot-and-Mouth Disease, P.O. Box 18021, Nairobi, Kenya.

MATERIALS AND METHODS

Viruses

The viruses used (Table 1) were passaged in BHK cells for the same number of times as were seeds being used to produce antigen in BHK suspension cultures.

Homologous antigens

Virus strains were grown in suspension culture for 48 hr., then harvested and cell debris removed by centrifugation at 6000 rev./min. for 2 hr. at 4° C. The cell-free virus suspension was inactivated with acetylethyleneimine at a final concentration of 0·05 % (v/v) for 30 hr. at 26° C. The inactivation procedure was stopped by adding sodium thiosulphate to a final concentration of 2 % (w/v). The suspension was then tested for live virus by injecting 0·03 ml. intraperitoneally into each of 50 'P' strain mice aged 4–7 days (Skinner, 1951) and also by inoculation of 10 ml. of the suspension onto each of six BHK monolayers.

Table 1. *Viruses used in the investigation, showing passages before use in cattle and in BHK cells*

		Number of passages		
			BHK	
Type	Strain (origin)	Cattle	Monolayer	Suspension
Asia 1	Pak. 1/54	0	13	1
C	B.F.S. 997/53	6	6	1
A	5. Ger. (Eystrup) European vaccine strain	5	5	1
O	1 B.F.S. 1860/67	1	5	1
SAT 1	5. (S.A. 13/61)	2	5	1

Cattle

Two-year-old cross-bred Devon steers were used. These were fully susceptible to FMD. They were housed in loose boxes so designed that the animals could be watered and fed without an attendant having to enter the box. Every precaution was taken to prevent accidental spread of infection during an experiment.

BHK cell monolayers

These were 2-day-old monolayers of BHK 21, clone 13, cells grown in Roux flasks or in 8 oz. medical flats.

RESULTS

(A) *Studies of the* in vitro *tissue culture test*

(1) *The effect of volume of inoculum on sensitivity*

Sets of ten BHK monolayers in 8 oz. medical flats (cell sheet area 55 cm.^{2}) were inoculated with 1, 8 or 20 ml. of a dilution in phosphate-buffered saline (PBS) of

a suspension of Asia 1 virus. Two experiments were done: in the first there were four ten-fold dilutions from 10^{-7} to 10^{-10}; in the second there were five three-fold dilutions from 10^{-7} to 10^{-9}.

The results (Table 2) were analysed using the logit transformation of the proportions of monolayers in which CPE was seen and constants were fitted for a linear effect of log. (dilution), a linear effect of log. (volume of inoculum), and for the mean difference between experiments. The statistical method is an iterative one and the computer programme for this type of analysis was described by Lewis (1968). The linear effects are the slopes of the two log. dose–response relationships averaged over the two experiments. If increased CPE associated with increased volume of inoculum were caused solely by the extra amount of virus, the slope of the dose–response line for volume of inoculum should equal that for dilutions. The values obtained were $1{\cdot}39 \pm 0{\cdot}22$ and $1{\cdot}40 \pm 0{\cdot}18$, respectively.

Table 2. *Numbers of BHK monolayers (out of 10) showing cytopathic effect due to Asia virus in relation to dilution of virus suspension and volume of inoculum*

	Volume of inoculum (ml.)					
	First experiment			Second experiment		
Log_{10} dilution of virus	1	8	20	1	8	20
−7·0	4	7	10	8	10	10
−7·5	—	—	—	5	10	10
−8·0	1	7	6	2	9	10
−8·5	—	—	—	2	5	7
−9·0	0	1	0	0	0	4
−10·0	0	0	0	—	—	—
Total	5	15	16	17	34	41
CPE (%)	12·5	37·5	40·0	34·0	68·0	82·0

Consequently, the increases in percentage of monolayers showing CPE associated with increasing volume of inoculum (Table 2) are accurately predictable from the changes associated with the dilution series. Thus, although the average depth of inoculum over the cell sheet area of about 55 cm.^{2} ranged from about 0·18 mm. for 1 ml. inoculum to 3·64 mm. for 20 ml. inoculum, this seemed not to modify the effect of the virus.

The expected mean percentage of monolayers showing CPE, calculated after fitting the three constants mentioned above, was 36·3 for the first experiment and 56·3 for the second. If the potency of the virus is assumed constant, this demonstrates significant variation in the sensitivities of different batches of BHK cells.

(2) *Effect of the presence of large amounts of inactivated homologous antigen*

The possibility that inactivated antigen might prevent the detection of small amounts of infective virus by a cellblocking or interference effect was examined. Serial dilutions of the viruses of types C, A and SAT 1, detailed in Table 1, were made in PBS and compared by tissue culture on sets of ten BHK monolayers, with

dilutions of the same viruses in their homologous antigens. Type A dilutions in homologous antigen produced significantly greater CPE than the corresponding dilutions in PBS, whereas types C and SAT 1 did not. The experiment with type A was repeated twice, with results similar to the first experiment. Table 3 summarizes the results of these experiments.

The essential conclusion relevant to innocuity testing is that there was no indication that homologous antigen might appreciably inhibit the detection of live virus on BHK monolayers. The results with type A virus, although inconsistent with those for the other types, were consistent with this conclusion.

Table 3. *Comparison of the CPE on BHK monolayers of C, A and SAT 1 viruses when diluted in homologous antigen, with their CPE when diluted in phosphate-buffered saline (PBS)*

(Data are numbers of monolayers (out of 10) showing CPE, except where indicated.)

Log_{10} dilution of virus	C		A (total of 3 experiments. No. of monolayers out of 30)		SAT 1	
	Antigen	PBS	Antigen	PBS	Antigen	PBS
−6·0	10	10	—	—	—	—
−6·5	—	—	30	28	—	—
−7·0	7	7	30	18	5	6*
−7·5	—	—	26	8	2	7
−8·0	1	0	18	3	0	0
−8·5	—	—	6	1	1	1
−9·0	0	0	2*	0*	—	—
Total	18	17	112	58	8	14
CPE (%)	45·0	42·5	70·5	36·3	20·0	35·9

* Out of 9 monolayers.

(3) *The effect of sub-culture*

Transfer of fluids from incubated cultures with no evidence of virus to fresh cultures may improve the efficiency of virus detection.

Dilutions of types A, O, C and SAT 1 were made in PBS and 8 ml. of each dilution inoculated onto ten (occasionally nine) BHK monolayers. Virus was adsorbed at room temperature for 30 min., 40 ml. maintenance medium were added and the monolayers incubated at 37° C. After 48 hr. incubation, 8 ml. of the supernatant medium was withdrawn from all cultures apparently still free of CPE and used to inoculate a fresh monolayer. These sub-cultures were treated similarly and fluids from those apparently free from virus were transferred to a further set of sub-cultures. Both primary cultures and sub-cultures were examined for CPE up to 72 hr. after inoculation. An additional test, following this scheme, was done with type C virus diluted in homologous antigen.

Table 4 shows that the first sub-culture revealed additional evidence of virus in all five experiments. This indicates that some of the original cultures received

Table 4. *Results of titrating five virus suspensions on BHK monolayers and of sub-culturing from monolayers showing no CPE at the primary culture*

(Monolayers with CPE/monolayers tested.)

Log_{10} dilution	Type C (antigen)		Type C (buffer)		Type SAT 1 (buffer)		Type O (buffer)		Type A (buffer)	
	Parent	First passage	Parent	First passage	Parent	First passage	Parent	First passage	Parent	First passage
−5	10/10	—	—	—	—	—	—	—	—	—
−6	10/10	—	10/10	—	10/10	—	7/10	2/3	—	—
−6·5	—	—	—	—	—	—	—	—	8/10	2/2
−7	6/10	1/4	5/10	5/5	9/10	0/1	0/10	2/10	6/10	2/4
−7·5	—	—	—	—	—	—	—	—	1/10	0/9
−8	1/10	0/9	0/10	0/10	1/9	1/8	1/10	0/9	0/10	1/9
−8·5	—	—	—	—	—	—	—	—	0/10	0/10
−9	0/10	0/9	0/10	1/10	0/9	0/9	0/10	0/10	0/10	0/10
−10	—	—	0/10	0/10	0/9	0/9	0/10	0/10	—	—

too little virus to cause obvious CPE but enough to cause infection and by the first sub-culture there was sufficient virus to infect enough cells to produce visible CPE. The second sub-culture never showed evidence of additional virus.

The practical conclusion, from the point of view of innocuity tests, is that a single sub-culture of BHK monolayers would help to detect minimal quantities of live virus.

(B) *Comparison of cattle tongue inoculation with BHK tissue culture tests*

Two series of investigations were made. The first examined the ability to detect live virus (types Asia 1, C, A and O) in tissue culture at dilutions at or beyond the extinction point for infectivity in cattle. The second compared the ID 50 for cattle and for BHK monolayers.

(1) *The response of tissue cultures to virus causing low levels of response in cattle*

The object of this work was to determine whether evidence of virus could be obtained from tissue cultures at dilutions that caused no lesions in cattle.

The dilution expected to cause 50 % cattle tongue lesions was taken as a starting point and a stepwise series of larger dilutions prepared, using inactivated homologous antigen as the diluent. Each dilution was inoculated intradermally into 25 sites on each of four cattle, using 0·1 ml./site. The series was extended until a dilution was reached that caused no lesion in any of the 100 sites injected. The full series of dilutions was cultured simultaneously on BHK monolayers. For virus types Asia 1, C and O, 20 ml. of each dilution were added to each of two 4-day BHK monolayers in Roux flasks. After 30 min. at room temperature, 80 ml. of maintenance medium were added and the flasks incubated at 37° C. for 48 hr. 20 ml. of medium from each negative culture were then transferred to a further two Roux flasks which were treated in the same way as the parent flasks. All cultures were examined daily for CPE for 3 days. Type A dilutions were added to 48 hr. BHK monolayers in 8 oz. medical flats, using 8 ml. on each of 10 monolayers/dilution. These were not sub-cultured.

Tissue culture fluid from all monolayers that showed CPE and from all control cultures that received inactivated antigen alone was injected intraperitoneally into 4–7-day-old '*P*' strain mice. Tissue homogenates from mice dying were tested for virus by micro-complement fixation.

All the dilutions used in the cattle tests produced CPE in BHK monolayers, either in parent cultures or first sub-cultures, and the presence of virus was confirmed by complement fixation. CPE was therefore observed at dilutions 10 to 100-fold greater than any that produced a lesion in cattle.

The cattle results (Table 5) being sufficiently extensive, the opportunity was taken to estimate the concentration of cattle infectious units for each virus, assuming the Poisson distribution, and to compare the observed with the expected numbers of lesions at each dilution.

If we denote the concentration in the parent suspension by m, then the concentration in any ten-fold dilution is $m_d = m/10^x$. The expected number of lesions

Table 5. *Observed numbers of lesions (out of 100) in cattle tests and expected numbers calculated on the assumption of a Poisson distribution of the frequency of infective doses in small samples*

Cattle

(*a*) Type Asia 1 virus

Log_{10} dilution	Observed number	Expected number	χ^2
−4·25	40	54·8	8·8
−4·5	33	36·0	0·3
−5·5	17	4·3	
−6	14	1·4	99·6
−6·5	0	0·4	
−7	0	0·1	

(*b*) Type C virus

−3·35	54	72·5	17·1
−3·6	28	51·6	22·3
−4·6	21	7·0	30·1
−5·1	10	2·2	
−5·35	14	1·2	
−5·6	3	0·7	559·5
−6·1	28*	0·5	
−7·1	1	0·0	
−8·1	0	0·0	

* Out of 200.

(*c*) Type O virus

−4·7	19	22·7	0·8
−5·7	7	2·5	
−6·7	0	0·3	6·3
−7·7	0	0·0	

(*d*) Type A virus

−6·2	19†	26·0	2·9
−7·2	13	4·1	
−8·2	0	0·4	15·4
−9·2	0	0·0	

† Out of 75.

out of n sites is then $n(1-e^{-m_d})$. If r is the observed number of lesions and R the expected number, goodness of fit can be tested by

$$\chi^2 = \frac{n(r-R)^2}{R(n-R)},$$

summed over all dilutions after merging neighbouring results with expectations less than 5 or greater than 95.

These calculations (Table 5, last column), based on maximum likelihood estimates of m, show gross deviations from the expected numbers of lesions in cattle tests. There were far too few lesions at the smaller dilutions and far too many at the larger.

Probably several factors contributed to the gross discrepancy from Poisson

expectation in the cattle test: (1) the dose remaining at the injected site after the needle has been withdrawn may vary considerably; (2) the sensitivity of the cattle to the virus may vary; (3) observers may tend to underestimate the number of independent reactions when lesions from many sites coalesce.

None of the tissue culture experiments produced suitable results for testing goodness of fit to the Poisson expectation, partly because the numbers of monolayers at each dilution were small and partly because dilutions were too widely spaced. However, the results of the second experiment of Table 2 were used and the total χ^2 (with 10 D.F.) for deviations from Poisson expectation was 6·37. This gives no indication of departure from Poisson expectation and we may therefore expect the theoretical relationship of size of test to probability of failure to detect live virus to correspond more closely to the practical situation in tissue culture than in cattle tests.

(2) *Comparative infectivity assays using cattle and tissue culture tests*

Five dilutions of types Asia 1, C, O and A virus were prepared in PBS and 0·1 ml. of each dilution was injected into five sites on the tongue of each of four cattle,

Table 6. *Comparative assays of four virus types on cattle and on BHK monolayers*

(*a*) Number of lesions (out of 25) on tongues of each animal in cattle test

Virus type	Dilution series (steps of 10^{-1})	Cattle 1	2	3	4	Total	Log_{10} ID 50/ml.
Asia 1	10^{-3} to 10^{-7}	3	11	15	15	44	5·72
C	10^{-2} to 10^{-6}	13	11	11	1	36	4·23
O	10^{-2} to 10^{-6}	5	12	7	12	36	4·30
A	10^{-2} to 10^{-6}	17	14	16	17	64	5·71

(*b*) Numbers of BHK monolayers (out of 60) with CPE in tissue culture tests

Virus type‡	Dilution series (steps of $10^{-0·3}$)	Cell batches 1	2	3	4	5	Log_{10} ID 50/ml.
C	10^{-7} to $10^{-8·5}$	14	12	5	18*	20*	6·05
O	$10^{-6·3}$ to $10^{-7·8}$	19	26	15	18	—	5·77
A	$10^{-6·7}$ to $10^{-8·2}$	13	24	27	20†	41†	6·51

* Dilution series steps of 10^{-1} from 10^{-6} to 10^{-9}; responses out of 40.
† Dilution series steps of $10^{-0·5}$ from $10^{-6·5}$ to 10^{-9}.
‡ Data for the Asia 1 type are given in Table 2.

(*c*) Comparison of log_{10} ID 50s estimated from cattle and tissue culture tests

Virus type	Log ID 50/ml. Cattle	Tissue culture	Ratio of ID 50s (Tissue culture/cattle)
Asia 1	5·72	7·24	33
C	4·23	6·05	67
O	4·30	5·77	30
A	5·78	6·51	6
			Weighted mean 40

making 25 injections/tongue (Henderson, 1949). This arrangement shows the dose–response relationship within animals and allows a form of analysis that takes account of sensitivity variation from animal to animal.

Dilutions in PBS of the same viruses were assayed on batches of BHK monolayers, using 10 monolayers/dilution and 8 ml./monolayer (the data of Table 2, when 1 and 20 ml. inoculum were also included, are used for the Asia 1 assay).

Each assay of each virus was analysed by fitting constants for animals (in the cattle tests) or for batch of BHK cells and for a linear log. dose–response relationship, using the logit transformation (Lewis, 1968). Results (Table 6) were converted to $\log_{10}$ ID 50/ml. and show that, in these experiments, the tissue culture assay was from 6 to 67 times as sensitive as the cattle assay.

Hyslop & Skinner (1964) provide evidence that the cattle tongue test grossly underestimates the amount of virus in a suspension because material is lost from the injection site. They found that responses to 0·005 ml. were similar to responses to 0·1 ml. The ratio of these doses is of the same order as the ratio of the cattle to BHK infectivity assay results. This suggests that there may be little difference between the sensitivities of cattle tongue epithelium and BHK cells; the difference in sensitivity between the systems probably results mainly from defects in the mechanics of tongue injection.

Because the sensitivities both of cattle and of batches of BHK cells vary, differences in the results obtained by the two methods depend in part on the particular cattle and cell batches used in the comparison. However, the variation between cattle was much greater than the variation between cell batches; nevertheless, the variation between cell batches is large enough to indicate that in virus assays the inclusion of a standard preparation would be advantageous.

(C) *Calculation of an appropriate volume of vaccine for innocuity testing on BHK monolayers*

In practice, a tangible risk must be taken because the volume of vaccine tested must be a small proportion of the amount produced in any batch. Given the volume of a batch (N ml.), an assumed number of infective ml. doses in the batch (m) and an acceptable probability of failing to detect this amount of infection (P), the amount to be tested (n ml.) can be calculated by solving the equation:

$$\frac{(N-m)!\,(N-n)!}{N!\,(N-m-n)!} = P.$$

Solutions can be obtained, using Stirling's approximation to the logarithms of the factorials, for given values of N, m and P. Tables containing 96 solutions (Table 7) have been obtained by means of a computer programme that starts with trial values of n and rapidly converges to the solution. The columns of the Table give solutions for fixed values of $p = m/N$, which are assumed proportions of infective doses. When the sample for testing is a small proportion of the batch (say 1 % or less), the values in Table 7 are very close to $-(\log_e P)/p$, which is the solution for n in the formula $e^{-np} = P$; this formula assumes Poisson distribution of infective doses in samples of size n when the sampling fraction is negligible.

Table 7. *Size of sample (ml.) from a batch of N l. of vaccine required for probability P of failing to detect a proportion p of 1 ml. infective doses*

$P = 0{\cdot}10$								N(1)
45	75	223	434	535	697	1000	1750	4
45	76	228	457	570	759	1134	2236	40
45	76	229	459	574	765	1147	2288	200
45	76	229	459	574	766	1150	2300	2000
$P = 0{\cdot}05$								
58	97	287	555	682	882	1248	2108	4
58	98	297	593	740	985	1469	2885	40
58	98	298	597	746	995	1491	2972	200
58	98	298	598	747	997	1496	2992	2000
$P = 0{\cdot}01$								
89	148	433	821	999	1273	1749	2734	4
90	151	456	908	1133	1504	2235	4348	40
90	151	458	917	1146	1527	2287	4550	200
90	151	458	919	1149	1532	2299	4598	2000
p 0·05	0·03	0·01	0·005	0·004	0·003	0·002	0·001	—

Inspection of Table 7 suggests that with a sample of 500 ml. there will be a reasonably small risk of failing to detect live virus. It can be seen that interpreting the results of such a test (in which no virus was detected) involves a statement about the values of both P and p, and that different pairs of values can be attached to the same test. Since $p = -(\log_e P)/500$ closely approximates the relationship of P to p, it is clear that whatever value of p is selected, there is a corresponding value of P, so that to fix either p or P is arbitrary. Table 8 shows

Table 8. *A selection of permissible interpretations of a negative innocuity test with 500 ml. of vaccine in terms of the probability of failing to detect a proportion of infectious doses*

Probability of failing to detect infection	Proportion of infectious 1 ml. doses/l.
0·2	3·2
0·1	4·6
0·05	6·0
0·02	7·8
0·01	9·2

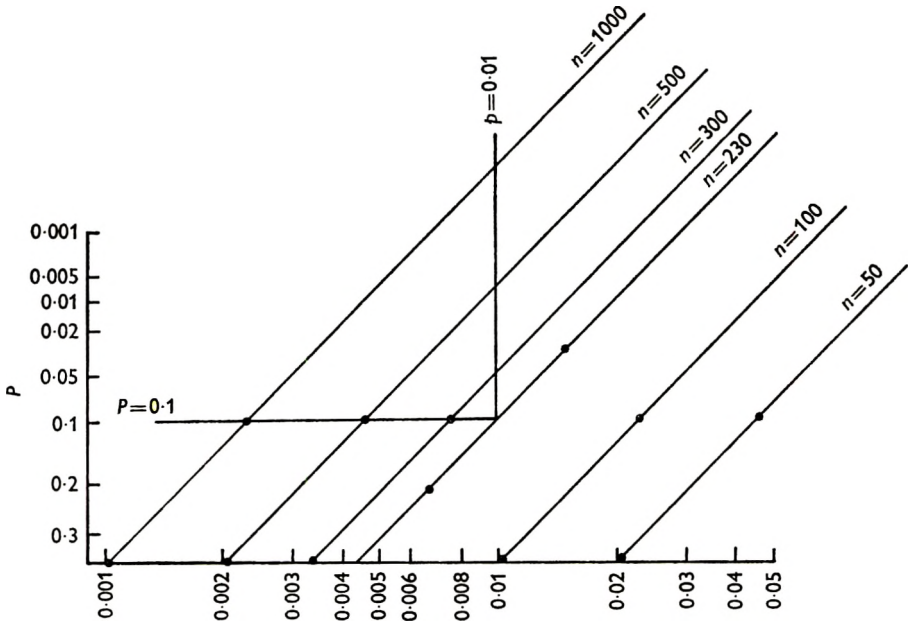

Fig. 1. Relationship of sample size (n) to probability (P) of failing to detect a proportion (p) of infective doses.

some corresponding values, with p converted to infectious doses/l. Thus, when a test of 500 ml. shows no evidence of virus, there is a 20% chance that the batch contained 3·2 infectious doses/l., or a 10% chance that it contained 4·6, and so on. Having fixed n, we can draw a curve relating P and p, but the choice of any point on the curve as an interpretation of the test is quite arbitrary. To simplify statements about the degree of confidence in a test, it may be useful to set a low limit

of acceptability at $P = 0{\cdot}1$ and $p = 0{\cdot}01$. A test of $n = 230$ satisfies this requirement. The ratio of any selected value of n to 230 is then a measure of the degree of confidence in the selected test relative to the arbitrary standard. The interrelationships of n, P and p are shown in Fig. 1, where equal intervals on the ordinate are $\log_e(-\log_e P)$ and those on the abscissa are $\log_e p$.

DISCUSSION

For a given number of units used in an innocuity test, the relation of P to p is calculated from a mathematical model, assuming that batches showing any evidence of virus will be rejected. We have selected a hypergeometric model as the most realistic, partly because it takes into account the size of batch being sampled but also because it assumes that the material consists of a set of units indistinguishable from each other except that some will infect the test system and some will not. The latter assumption is also implicit in the binomial model used by Henderson (1952*b*). Poisson-type models assume that the material contains a number of randomly distributed dimensionless infective particles. The Poisson model is a reasonable representation of contaminating virus particles in a vaccine; in the present context the particles are merely notional, for the test systems provide no means of counting them. For small p, calculations from the present hypergeometric model differ appreciably from those given by binomial or Poisson models only when the size of sample to be tested is an appreciable proportion of the batch.* Only a very special situation could justify such a large sample.

In most practical situations, calculations based on the Poisson model $n = -\log_e P/p$, as represented in Fig. 1, are sufficiently correct. For the cattle tongue test, we (following Henderson, 1952*b*) take p to represent a proportion of infective 0·1 ml. doses that a test of n doses of 0·1 ml. might fail to detect, with probability P. To find the number of 0·1 ml. doses required to give probability P of failing to detect the same proportion of vaccination doses, each consisting of $k \times 0{\cdot}1$ ml., we merely substitute kp for p in the formula, to get $n = -\log_e P/(kp)$. This is a well known result in Poisson theory. Thus, $n' = nk$ is the number of 0·1 ml. doses to be tested and, if the vaccination dose is 1 ml., $n' = 10n$. In general, if theoretical predictions are to be stated in terms of a proportion p of infected vaccination doses, then $n = -\log_e P/p$ is the number of vaccination doses to be included in the test. This is a simple generalization of results presented in tabular form by Henderson (1952*b*, Tables 7, 8).

The cost of testing more than 15 ml. of vaccine on cattle is prohibitive. This size of test corresponds to a 1/20 chance that 18 % of 1 ml. vaccination doses might contain amounts of live virus detectable on cattle tongues. Although no outbreaks of infection following the use of vaccines that passed the cattle test have been

* A referee has pointed out that $P = (N - n^m/N)$ gives very similar results to the hypergeometric model and is readily solved by taking logarithms. This relationship was the basis of our method of solving the hypergeometric equation but we overlooked the fact that in itself it is a valid solution to the problem. Our attention has also been drawn to a paper by Peto & Maidment (1969) dealing with a very similar problem, in which they use a formula very similar to the formula of this footnote.

reported, the theoretical risk is far from negligible. With the tissue culture method, the amount of vaccine tested can be selected to make the theoretical risks very small. The method cannot be used after the vaccine has been prepared for field use, because of the cytotoxic effect of certain adjuvants; at this stage, there may be a case for a cattle test to check against accidental gross contamination with live virus of batches that passed the tissue culture test.

The results of Hyslop & Skinner (1964) suggest that the apparent difference in susceptibility between cattle tongue epithelium and BHK cells (Table 6) may be mainly a consequence of the loss of injected virus from sites injected in cattle tongues. Variation in this loss should be one of the factors causing variation in the relative sensitivity of the two assay methods shown in Table 6 (*c*). The loss would also affect calculations of the relative sensitivity of the intradermal tongue and subcutaneous routes of injection made by Henderson (1952*a*). Thus, the subcutaneous route may well have about a tenth of the sensitivity (relative to cattle tongue epithelium) previously supposed. This becomes an additional safety factor in subcutaneous vaccination.

The demonstration in Section B 1 that responses to the cattle test showed gross deviations from Poisson expectation, whereas responses to the tissue culture test did not, implies that theoretical calculations of probabilities of failing to detect infection should be much nearer the truth for tissue culture than for cattle tests.

Our results show that BHK tissue cultures are satisfactory for use in detecting small amounts of infective virus remaining in incompletely inactivated FMD virus suspensions. The inactivated antigen does not interfere appreciably with the detection of residual virus, though at least one sub-culture of the material under test is needed to be certain of obtaining visible CPE. A reasonable volume of inoculum can be applied to each cell sheet, so that fairly large volumes of antigen can be tested on a manageable number of BHK monolayers. This ability to test large sample volumes is the real advantage in the use of a tissue culture test and gives greatly improved levels of confidence compared with the cattle test.

Gard (1960) has argued that there is an implicit advantage in the safety testing of material at various stages of production. This was too costly with the cattle test but it becomes practicable with a tissue culture test. Such tests could be done on each batch of antigen produced and again on multivalent mixtures of antigen. Antigens passing these tests could then be removed to a 'clean' area for final formulation. Tissue culture tests cannot be used after this stage because of the cytotoxic effects of formulation adjuvants.

REFERENCES

GARD, S. (1960). Theoretical considerations in the inactivation of viruses by chemical means. *Annals of the New York Academy of Science* **83**, 638.

HENDERSON, W. M. (1949). The quantitative study of foot-and-mouth disease. *Agricultural Research Council Report Series*, no. 8. London: Her Majesty's Stationery Office.

HENDERSON, W. M. (1952*a*). A comparison of different routes of inoculation of cattle for detection of the virus of foot-and-mouth disease. *Journal of Hygiene* **50**, 183.

HENDERSON, W. M. (1952*b*). Significance of tests for non-infectivity of foot-and-mouth disease vaccines. *Journal of Hygiene* **50**, 195.

HYSLOP, N. & SKINNER, H. H. (1964). A note on the interpretation of infectivity titres of foot-and-mouth disease virus based on cattle tongue titrations. *Bulletin de l'Office International des Épizooties* **61**, 1091.

MACPHERSON, I. A. & STOKER, M. G. P. (1962). Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. *Virology* **16**, 147.

LEWIS, J. A. (1968). A program to fit constants to multiway tables of quantitative and quantal data. *Applied Statistics* **17**, 33.

PETO, S. & MAIDMENT, B. J. (1969). Tables of the upper limit to the estimate of the density of contaminating particles in a medium. *Journal of Hygiene* **67**, 533.

SKINNER, H. H. (1951). Propagation of strains of foot-and-mouth disease virus in unweaned white mice. *Proceedings of the Royal Society of Medicine* **44**, 1041.

J. Hyg., Camb. (1970), **68**, 173
Printed in Great Britain

The relevance of the anti-human globulin (Coombs) test and the complement-fixation test in the diagnosis of brucellosis

By D. G. McDEVITT

Department of Therapeutics and Pharmacology, Queen's University of Belfast

(*Received* 4 *November* 1969)

SUMMARY

The relationship between the serological findings for brucellosis and the epidemiological factors has been studied in veterinary surgeons in Northern Ireland. The anti-human globulin (Coombs) test and the complement-fixation test for brucella were used in two groups of veterinary surgeons, those self-employed and those employed by the Ministry of Agriculture.

Significant serological differences were found to exist between the two groups. Those in private practice showed changes related to age, cattle skin rash, reactions to S. 19 vaccine accidents and symptoms suggestive of brucellosis in the past or the last year. But those working for the Ministry only showed titre changes related to the length of their private practice experience before joining the Ministry. In neither group was there a relationship between serological findings and the type of milk drunk or any particular group of symptoms suggestive of brucellosis.

The findings indicate that high titres to brucella by the Coombs and complement-fixation test can occur in people repeatedly exposed to infection at work. Titres which would be of diagnostic importance in the rest of the population may be of little diagnostic significance even when they are as high as 160 Coombs and 128 complement fixation.

INTRODUCTION

The difficulties of diagnosing brucellosis in the absence of a positive culture of *Brucella abortus* from the patient's blood or other tissues are well known. The direct agglutination test has been generally agreed to be the most useful laboratory aid when cultures are negative (Evans, Robinson & Baumgartner, 1938; Spink, 1956; Dalrymple-Champneys, 1960; Joint FAO/WHO Expert Committee on Brucellosis, 1964), but unfortunately it has its limitations and its interpretation is not universally agreed.

Recently attention has been turned to the immunoglobulin pattern of antibodies against brucellas in both man and animals, and this has rekindled interest in the anti-human globulin (Coombs) test and the complement-fixation test for brucellosis. The latter test measures IgG antibody (Heremans, Vaerman & Vaerman, 1963; Reddin, Anderson, Jenness & Spink, 1965); and, in the absence of agglutination, the Coombs test also measures IgG, together with IgA. The presence

of IgG-type immunoglobulins has been thought to be the distinguishing feature of active human infection (Reddin *et al.* 1965; Kerr, Coghlan, Payne & Robertson, 1966*b*; Macdonald & Elmslie, 1967).

Using the direct agglutination test, the anti-human globulin (AHG) test and the complement-fixation (CF) test, Kerr, Coghlan, Payne & Robertson (1966*a*, *b*) concluded that symptoms and laboratory findings could be correlated to make a diagnosis of chronic brucellosis in a patient. If the tests were negative the diagnosis was excluded. Macdonald & Elmslie (1967) agreed with these findings.

Meanwhile it was pointed out that, because agglutination titres as high as 180 were not uncommon in some rural populations in apparently healthy people, it would be wise to examine a series of several thousand 'normals' by the AHG and CF tests before too much weight was given to these methods (Lancet, 1966).

A general serological survey for brucellosis has been carried out in Northern Ireland using these tests, together with the direct agglutination test (McDevitt & McCaughey, 1969). It showed that the serological prevalence of human brucellosis in the province was largely confined to occupational groups working with cattle. This paper records a more detailed study of the serological findings in one of these occupational groups. An attempt is made to determine the epidemiological factors which lead to the development of high antibody titres amongst veterinary surgeons and the relationship between these factors, the antibody titres and the clinical diagnosis of active brucellosis.

METHODS

Every veterinary surgeon in private practice in Northern Ireland in 1966 was sent a questionnaire about practice habits, amount of exposure to cattle, use of *Br. abortus* Strain 19 vaccine, milk drinking habits, previous history of brucellosis, skin rashes and past or present symptoms, which might be related to infection with *Br. abortus*.

If any reply suggested a previous or present diagnosis of brucellosis, or reported symptoms which might have been consistent with such a diagnosis, the person concerned was visited at his home. A more detailed history, both of epidemiology and of illness, was obtained and a general physical examination was carried out.

A sample of serum was sought from all practitioners for estimation of AHG and CF titres of antibody to *Br. abortus*. Each specimen was submitted for laboratory assessment under a code number, as the veterinary surgeons and some of their clinical histories were known to their colleagues in the Veterinary Research Laboratories.

For comparison the veterinary surgeons employed by the Ministry of Agriculture in Northern Ireland were also investigated. Although they are often in close contact with cattle in carrying out their duties in the tuberculosis and brucellosis eradication programmes, they are spared the most intimate contact which is associated with bovine midwifery. Their sera were tested by the same methods and a modified questionnaire was circulated to them. This omitted milk drinking history, but included questions on length of private practice before entering the Ministry, duration of employment by the Ministry and nature of present duties.

Laboratory methods

Anti-human globulin (Coombs) test

This was carried out by the method of Wilson & Merrifield (1951) as modified by Kerr *et al.* (1966*b*). The antigen used was an agglutinable suspension of *Br. abortus* Strain 99 standardized to give 50 % agglutination with a 500 dilution of International Standard Serum. Anti-human precipitating rabbit serum (Burroughs-Wellcome) was used at its optimal dilution.

Complement-fixation test

This test was carried out as described by Bradstreet & Taylor (1962), using a 4 volume test (unit volume 0·1 ml.) in W.H.O. plastic plates. The short fixation method only was used with 2·0 M.H.D. of complement and the optimal dilution of antigen (heat-killed *Br. abortus* Strain 99) as determined by a chessboard titration. In each series of tests a known control serum was included.

Statistical methods

In comparison of the various groups separated by the epidemiological and clinical factors, the χ^2 test was used when the numbers were sufficient. When the numbers were too small for the valid application of χ^2, the exact double tail probability test was used. In either case differences were considered significant when the probability (P) was less than 0·05 (1/20).

RESULTS

Populations

There were 125 veterinary surgeons in private practice in Northern Ireland in 1966, when this study was commenced. A total of 116, or 92·8 %, were included in the investigation; of the nine excluded, no blood sample could be obtained from five, no questionnaire from two and no information at all from the remaining two.

The veterinary surgeons employed by the Ministry of Agriculture are a more complex group, totalling 108. Twenty-seven of these were engaged in entirely administrative occupations with no field contacts, and sera were obtained from only nine of them. Three worked in artificial insemination plants. The remaining 78 were engaged in field work, consisting mainly of brucellosis and tuberculosis eradication in cattle and meat inspection, and it was possible to obtain information from 64 or 82·1 % of this latter group.

In order to make the two main groups, private practice and Ministry practice, more homogeneous, those whose work did not bring them into contact with cattle to any extent were excluded, thus confining the private practice group to the 110 doing at least 50 % cattle work and the Ministry group to the 64 engaged in field work. There was a suggestion that the excluded subgroups contained lower

* The detailed tables of the findings for each individual concerned in this analysis are not included in this paper. The tables printed to illustrate the text are representative and simplified examples of those used in the analysis. Those wishing more detailed figures should consult the author or his thesis (McDevitt, 1968).

titres for the AHG and CF tests but the numbers were not large enough to attain statistical significance when compared with the larger groups.

Serological findings in private and ministry practice

Tables 1 and 2 show the distribution of AHG (Coombs) and CF titres in the two groups of veterinary surgeons. The majority show raised titres by both tests but the titres tend to be higher in private practitioners than in those working for the Ministry.

Table 1. *Distribution of serological titres in veterinary surgeons in private practice*

(Analysis includes all samples tested for both AHG and CF titres)

	AHG (Coombs) titres				
CF titres	0 and 10	20 and 40	80 and 160	320 and over	Total
0 and 4	9	13	7	0	29
8 and 16	1	9	11	2	23
32 and 64	0	5	22	3	30
128 and 256	0	0	8	14	22
512 and over	0	1	0	2	3
Total	10	28	48	21	107

Table 2. *Distribution of serological titres in veterinary surgeons working for the Ministry*

(Analysis includes all samples tested for both AHG and CF titres)

	AHG (Coombs) titres				
CF titres	0 and 10	20 and 40	80 and 160	320 and over	Total
0 and 4	28	14	2	1	45
8 and 16	1	8	4	0	13
32 and 64	0	3	6	0	9
128 and 256	0	0	1	2	3
512 and over	0	0	0	0	0
Total	29	25	13	3	70

The difference is significant with both the AHG and the CF tests (see Table 3). Although the Ministry veterinary surgeons show a significantly greater number of raised titres compared with the general population (McDevitt & McCaughey, 1969) 50 % of them have negative CF tests. There is a general correspondence between the AHG titre and the CF titre, but the AHG titre is more frequently positive with a negative CF test than the reverse. There were 14 in the private and 20 in the Ministry group with raised AHG titres and negative CF tests whereas there were no private and only two of the Ministry group with positive CF tests and negative AHG titres. Because of this lack of direct relationship between the two tests they are considered separately in the following analysis, but raised titres in either test will be regarded as evidence of contact or infection in the subsequent discussion.

Private practice

Influence of the amount of cattle work

From the replies to the questionnaire it was possible to divide these 110 into 53 who spent more than 75% of their work with cattle and 57 who only worked from 50 to 75% of their time with cattle. Although the titres were on the average higher amongst those doing over 75% cattle work the difference was not statistically significant.

Table 3. *Serological findings in private practice and Ministry groups of veterinary surgeons, excluding those not in frequent contact with cattle*

	AHG (Coombs) titre				
	⩽ 10	20 and 40	80 and 160	> 160	Total
Private	8	27	52	23	110
Ministry	24	23	14	3	64

On ungrouped data: $\chi^2 = 36{\cdot}54$. D.F. = 5. $P < 0{\cdot}001$.

	Complement-fixation titre				
	Nil	4 and 8	16 and 32	> 32	Total
Private	19	15	31	36	101*
Ministry	34	9	13	5	61*

On ungrouped data: $\chi^2 = 28{\cdot}6$. D.F. = 5. $P < 0{\cdot}001$.
* Insufficient serum for testing nine private and three Ministry vets.

Table 4. *Effect of age on serological findings in veterinary surgeons in private practice*

	AHG (Coombs) titre			
	Age			
Titre	20–29	30–39	40–49	50+
40 or less	11	10	11	3
80 or more	7	23	33	12

$\chi^2 = 9{\cdot}06$. D.F. = 3. $0{\cdot}05 > P > 0{\cdot}02$.

	Complement-fixation titre			
	Age			
Titre	20–29	30–39	40–49	50+
8 or less	10	12	6	6
16 or more	6	17	35	9

$\chi^2 = 13{\cdot}64$. D.F. = 3. $0{\cdot}01 > P > 0{\cdot}001$.

Influence of age

The distributions of the AHG and CF titres with age in private practice are shown in Table 4. Most of these veterinary surgeons were between 30 and 50 years old. There was a significant difference in the distribution of the AHG titre between the age-groups which was largely accounted for by the high proportion of the 20–29 year group in the low titre range and the corresponding low proportion in the high titre range. With the CF test, the main difference was found in the 40–49 year age-group, most of whom had titres of 16 or greater.

Because of this relationship between age and titre using these two tests it was necessary to rule out the possibility that age was significantly related to the other factors examined.

Milk drinking habits

Eighty of the 110 veterinary surgeons in private practice drank pasteurized milk. The remaining 30 drank either raw or a mixture of raw and pasteurized milk. There was no evidence that milk drinking habits were related to age.

Milk drinking habits had no significant influence on the distribution of the AHG titres or on the CF titres.

Cattle skin rash

Those replies which appeared to be describing sensitivity to the various cleansing materials used in obstetrical and other work were ignored. Many of the rest were

Table 5. *Relation between cattle skin rash and serological findings in veterinary surgeons in private practice*

	AHG (Coombs) titre		
	0 to 20 incl.	40 to 160 incl.	Over 160
Rash	2	32	11
No rash	20	33	12

$\chi^2 = 11{\cdot}51$. D.F. = 2. 0·05 to 0·001.

	Complement-fixation titre		
	8 or less	16 and 32	> 32
Rash	11	13	18
No rash	23	18	18

$\chi^2 = 2{\cdot}3$. D.F. = 2. $0{\cdot}2 > P > 0{\cdot}1$.

the sequelae of manual removal of retained placentas in cattle. Forty-five veterinary surgeons in private practice developed these cattle skin rashes, 65 did not. Again, there was no significant relationship between the age-groups and the occurrence of cattle skin rashes.

Table 5 shows the AHG and CF titre distributions for those with and without rashes. Those with cattle skin rashes differed significantly from those without

rashes in the distribution of their AHG titres. The difference was composed primarily of the very small proportion of people with rashes who had titres of 20 or less, and a correspondingly larger proportion with titres of 80. In the group with no rash more than expected had titres of less than 40. The CF test showed no significant difference between the distributions of the two groups.

The use of Brucella abortus *strain* 19 *vaccine* (*S.* 19)

S. 19 vaccine is a live attenuated vaccine which is capable of producing infection in humans. One hundred and six of the veterinary surgeons had used the vaccine at some time; only four had never used it. Thirty-seven had had some sort of accident with S. 19 vaccine, either self-inoculation, ingestion or contamination of the conjunctivae: of these, 20 had experienced a subsequent reaction, either systemic or local, and 16 had not.

Table 6. *S.* 19 *vaccine accidents in relation to age and serological findings in veterinary surgeons in private practice*

	Age			
	20–29	30–39	40–49	50+
(*a*) Accidents admitted	3	9	17	8
Accidents denied	17	28	27	8

$\chi^2 = 7{\cdot}709$. D.F. = 3. $0{\cdot}1 > P > 0{\cdot}05$.

	AHG titre				
	0–20	40	80	160	Over 160
(*b*) Accidents admitted	3	6	9	8	11
Accidents denied	19	1	8	17	12

$\chi^2 = 6{\cdot}3$. D.F. = 4. $0{\cdot}2 > P > 0{\cdot}1$.

	Complement-fixation titre (40–49 age-group only)	
	0–16	Titre 32 and over
(*c*) Accidents admitted	1	14
Accidents denied	14	14

$\chi^2 = 8{\cdot}10$. D.F. = 1. $0{\cdot}01 > P > 0{\cdot}001$.

Table 7. *Reaction to a vaccine accident in veterinary surgeons in private practice* (*all ages*)

	A.H.G. (Coombs) titre			
	0–40	80	160	320 and over
Reaction to accident	2	3	5	10
No reaction or no accident	33	23	31	13

$\chi^2 = 9{\cdot}53$. D.F. = 3. $0{\cdot}05 > P > 0{\cdot}02$.

These accidents were admitted more frequently by the older than by the younger (see Table 6) but the difference was hardly significant. Taking the group as a whole the titres in both AHG and complement-fixation tests tended to be higher amongst those admitting to accidents but the differences were not significant. But taking the 40–49 age-group alone there is a significantly higher proportion with high complement-fixation titres amongst those admitting to vaccine accidents (Table 6).

When those admitting to local or systemic reactions to the accidents are compared with the rest the difference in AHG titre is significant irrespective of age (see Table 7). The figures for complement fixation are similar.

Therefore although there is evidence that these vaccine accidents, especially when followed by a reaction, may lead to high titres in the elderly, they are not the only factor.

Influence of a previous history of brucellosis

Thirty veterinary surgeons in private practice claimed to have a past history of brucellosis, though in none had the diagnosis been confirmed by blood culture. In many the diagnosis was self-made or was based on serology rather than symptoms.

The distributions of the serological titres of those claiming to have had brucellosis and those with no previous history showed no difference for either the AHG test or the CF test.

The frequency of a positive history for brucellosis was significantly higher amongst the 20–29-year-old veterinary surgeons than amongst the older men. As positive serological reactions are relatively uncommon among those in this age group it seems unlikely that many of these young men had actually had brucellosis.

The relationship of symptoms to serological tests

Initially it was intended to divide the veterinary surgeons into those with symptoms suggestive of active brucellosis, those with other symptoms and those who were asymptomatic, on the basis of the questionnaires and the clinical histories obtained. This clear distinction was not found possible because of the variety of symptom complexes, and the vagueness of the symptoms of which they complained. It was found that the impression of genuineness gained by the observer at interview seemed to carry most weight. Thus they were grouped first into those who complained of any of the symptoms about which enquiry was made —in fact, it was found that generally they did not report symptoms unless they were or had been persistent—and second, into those whose symptoms had occurred within the previous 12 months. A number complained of episodes of illness which occurred several times per year, often similar to influenza, but without seasonal variation.

Forty-eight veterinary surgeons in private practice admitted to symptoms, either past or present, amongst the ones suggested. Between them they complained of 216 symptoms, of which the most frequent were sweating (25 times), weakness (24), malaise (30), headache (20), irritability (20) and backache (25). Others commonly encountered were rheumatism (17) and depression (16).

There was no increase in frequency of symptoms with age nor were there markedly higher AHG or CF titres in those complaining of symptoms compared with those who did not. However, there was a significant higher proportion of those with symptoms with AHG titres of over 40 (Table 8). By confining the comparison to those who complained of symptoms during the last year against the rest

Table 8. *Relation between serological findings and symptoms suggesting brucellosis in veterinary surgeons in private practice*

	A.H.G. (Coombs) titre				
	0–20	40	80	160	> 160
Symptoms	6	4	14	13	11
No symptoms	16	9	12	13	12

For ungrouped data: $\chi^2 = 4{\cdot}97$. D.F. = 4. $0{\cdot}3 > P > 0{\cdot}2$.
Grouped 0–40 against > 40: $\chi^2 = 3{\cdot}88$. D.F. = 1. $0{\cdot}05 > P > 0{\cdot}02$.

Table 9. *Relation between serological findings and symptoms suggesting brucellosis in the previous year only, in veterinary surgeons in private practice*

	A.H.G. (Coombs) titre	
	0–80	> 80
Symptoms	9	16
No symptoms	52	33

$\chi^2 = 3{\cdot}99$. D.F. = 1. $0{\cdot}05 > P > 0{\cdot}02$.

	Complement-fixation titre	
	0–64	> 64
Symptoms	13	10
No symptoms	63	15

$\chi^2 = 4{\cdot}38$. D.F. = 1. $0{\cdot}05 > P > 0{\cdot}02$.

significant differences were shown for the higher titres of both AHG and CF titres (see Table 9).

The lack of a clear-cut relationship casts doubt on how much weight can be placed on these symptoms in the diagnosis of brucellosis.

Veterinary surgeons employed by the Ministry of Agriculture

It has already been shown (Table 3) that those employed by the Ministry of Agriculture had a much lower range of serological titres to *Br. abortus*, measured by both the AHG and the CF tests, than those in private practice.

Influence of age

In this group increasing age was not associated with a rising antibody titre to brucella by the AHG (Coombs) test (Table 10). The complement-fixation test

showed a high proportion of veterinary surgeons with titres of over 4. The majority of those over 40 years of age had titres of less than 4. This is in contrast to the findings amongst private practitioners, where both tests showed a tendency for the titre to rise with age.

Table 10. *Effect of age on serological findings in veterinary surgeons working for the Ministry*

	AHG			CFT		
	0–20	> 20	Total	0–4	> 4	Total
20–29	4	0	4	2	2	4
30–39	3	6	9	1	8	9
40–49	20	14	34	23	9	32*
50+	11	6	17	11	5	16*
	$\chi^2 = 0{\cdot}37$. D.F. = 2. $0{\cdot}9 > P > 0{\cdot}8$.			$\chi^2 = 9{\cdot}8$. D.F. = 2. $0{\cdot}01 > P > 0{\cdot}001$.		

* Serum insufficient for CF tests in three vets.

Table 11. *Effect of previous private practice on serological findings in veterinary surgeons working for the Ministry*

Private practice (yrs.)	AHG		CFT	
	0–20	> 20	Nil	Four or over
0–5	31	13	27	16
> 5	7	13	7	11
	$\chi^2 = 7{\cdot}164$. D.F. = 1. $0{\cdot}01 > P > 0{\cdot}001$.		$\chi^2 = 2{\cdot}93$. D.F. = 1. $0{\cdot}1 > P > 0{\cdot}05$.	

Table 12. *Effect of interval since finishing private practice on serological findings in veterinary surgeons working for the Ministry*

(Includes only those who spent more than 5 years in private practice)

Years since private practice	AHG (Coombs) titre		
	0–10	20	40 and over
< 10	0	1	8
> 10	5	1	5

Exact probability = 0·046.

Many of those working for the Ministry had spent some time in private practice before being employed by the Ministry. Table 11 shows that the duration of such private practice had a significant effect on the frequency of high titres by the AHG tests but not by the CF test.

The frequency of high complement-fixation titres amongst young Ministry veterinary surgeons might be due to their recent experience in private practice. Similarly long service with the Ministry could be associated with a fall in titre

with the increasing interval since serious exposure. Table 12 compares those with less than 10 years' Ministry service with those with more. All these had had at least 5 years in private practice and might be expected to have started with high titres. The results are consistent with a falling titre after more than 10 years' Ministry service but the numbers are too small to be quite significant.

The other data for the 64 veterinary surgeons employed in field work by the Ministry of Agriculture showed that nine of them had experienced cattle skin rashes; 15 of them had had some sort of S. 19 vaccine accident when in private practice and nine of these had experienced a subsequent systemic or local reaction. Only four gave a past history of having had brucellosis, and 14 of them complained of symptoms which might have been connected with brucellosis. None of these factors related significantly to the distribution of serological titres.

Comparison between the veterinary surgeons employed by the Ministry and those in private practice at the time of the survey showed several significant differences. More of the private practitioners gave a history of cattle skin rash and of having been diagnosed as brucellosis. Fewer Ministry veterinarians complained of symptoms (past or present) suggestive of brucellosis.

DISCUSSION

Spink (1951) considered that epidemiology should be taken into account in the interpretation of the results of laboratory tests and in the clinical diagnosis of brucellosis. He pointed out the relevance of age, sex and occupation in diagnosis. Elsewhere he suggested that most patients with culturally proven brucellosis had a direct agglutination titre of 320 or above (Spink, McCullough, Hutchings & Mingle, 1952). No comparable work has been reported using the AHG and CF tests, and it is in the patients from whom the infecting organisms are least likely to be obtained that these more sensitive tests may be most valuable.

This study in veterinary surgeons would indicate that a knowledge of the epidemiological history may be helpful in interpreting the serological brucella antibody findings in a person who is occupationally exposed to brucella infection. The titre of brucella antibodies in veterinary surgeons appears to be related to type of practice, age-group, experience with *Br. abortus* S. 19 vaccine, presence of skin rash when handling cattle and history of symptoms. The AHG titre relates to all the factors, whereas the CF titre distribution does not relate to either cattle skin rash or to symptoms, other than those occurring within the previous one year. The latter test may be more helpful, therefore, in symptomatic patients.

Veterinary surgeons who are in private practice are much more likely to have high antibody titres than those working for the Ministry. Those in private practice who are under 30 years of age are likely to have lower titres with both the AHG and CF tests than those 30 years of age or above. A high titre in a veterinarian under 30 years of age may be regarded as more significant than a similar titre in later years.

In addition, veterinary surgeons now working for the Ministry who had previously been in private practice for more than 5 years had higher antibody titres,

measured by the AHG test, than those who had worked for less than 5 years in private practice. After 5 years in private practice the antibody titres tended to be higher for those less than 10 years in the Ministry, than for those who had been more than 10 years in the Ministry. This would suggest that these tests, particularly the AHG test, are an index of exposure to *Br. abortus* infection and that 5–10 years in private practice are long enough for most to acquire brucella antibodies. The Ministry veterinarians' experience of the antigen cannot be confined entirely to their time in private practice as their unqualified assistants also show evidence of brucella antibodies (McDevitt & McCaughey, 1969). However, if the relationship between high titres and length of private practice is important, then it would appear that the high titres may persist for up to 10 years. This idea is in agreement with previous authors (Dalrymple-Champneys, 1929; Bartram *et al.* 1963), but, if true, it makes the interpretation of the relevance of antibody titres even more difficult.

Veterinary surgeons who had experienced a local or systemic reaction following accidental self-administration of *Br. abortus* strain 19 vaccine were more likely to have an AHG titre of 160 or greater or a CF titre of 128 than those who had not. This is important because the same titres may be found in the sera of those who have recent symptoms suggestive of brucellosis. There was no relationship between recent symptoms and S. 19 vaccine reactions. The Ministry of Agriculture in Northern Ireland is now rigidly controlling the use of S. 19 vaccine to calves between 4 and 8 months old, because its random use may make it difficult to interpret the serological tests in the eradication scheme. The S. 19 vaccine accidents may be expected to be much fewer in future.

The distribution of the AHG titre in this study was also related to skin rashes after handling the products of conception in cattle. Those who got such rashes were more likely to have a titre of 80 or greater than those who did not. Skin rash may be a manifestation of hypersensitivity to brucella antigen and some veterinarians claim the ability to diagnose brucellosis at parturition by putting an arm into the cow's vagina. If they develop a rash, then subsequent laboratory testing will be positive. Two veterinary surgeons in this series described this phenomenon, but their serological findings were no different from those of many others.

The most important question relates to symptoms. How can the AHG and CF tests help in the case of a patient with symptoms suggesting brucellosis who is also occupationally exposed? The symptoms associated with brucellosis are many, commonplace and often similar to those of psychoneurosis. In addition, many people in exposed occupations have evidence of brucella antibodies irrespective of the laboratory method used.

One factor which may be important in the production of symptoms is the type of work done. Private practice demands many hours of work during the day and often also at night, sometimes for 7 days per week, and the work is often heavy: Ministry work consists of a 40-hour week performing duties which are routine and less demanding physically. A significantly greater proportion of veterinary surgeons in private practice complained of symptoms than of those in Ministry employ. However, Henderson (1967) did not find 'rheumatism' and arthritis more

frequently in dairy farmers and other allied groups than in some groups unexposed to brucellosis, nor did he find these symptoms related to raised brucella antibody titres. Therefore, if physical effort is not important in the production of symptoms, the difference between the proportion of veterinarians in private practice and those in the Ministry complaining of symptoms may be the result of the amount of exposure to infection.

Again, among those engaged in private practice an AHG titre of 160 or greater and a CF titre of 128 or greater was found more often in those who complained of symptoms consistent with brucellosis than among those who did not so complain. If as suggested the tests are an index of exposure to brucella infection, then this is a further indication that symptoms are most likely in those who have the greatest exposure.

There are points of agreement between this latter serological finding and those of Macdonald & Elmslie (1967), who reported that, in a post-treatment group of patients with brucellosis, those who remained symptomless for more than one year after treatment had CF titres of less than 10 and AHG titres of less than 160.

It has previously been shown that, in Northern Ireland, people who are not occupationally exposed to brucellosis are unlikely to show brucella antibody titres of greater than 20 by either the standard agglutination or the AHG tests, or greater than 8 by the CF test (McDevitt & McCaughey, 1969). Higher serological titres in persons drawn from the general population, particularly in patients with symptoms or signs consistent with brucellosis, must suggest such a diagnosis—but the diagnosis must not be made uncritically and effort must be made to obtain some history of possible exposure, remembering that milk-borne infection in an occupationally unexposed patient now seems unlikely in Northern Ireland.

This present study would suggest that, in groups occupationally exposed to brucellosis, such as the veterinary surgeons in private practice, the situation is entirely different. The serological antibody titres must then be interpreted in the light of the epidemiological history—particularly the patient's age or duration of practice, occurrence of skin rashes on exposure to cattle, history of reaction following an S. 19 vaccine accident, and a history of past or present symptoms suggestive of brucellosis. Symptoms within the previous one year may be associated with a titre of 160 or greater with the AHG test and a titre of 128 or greater with the CF test. But an S. 19 vaccine reaction, no matter how long ago it occurred, may produce similar antibody titres.

In the group of veterinary surgeons under study, 38 % of those with no recent symptoms, and 40 % of those with no symptoms whatsoever, had an AHG titre of 160 or greater; the corresponding figures for the CF test at a titre of 128 or greater were 19 and 17 % respectively. If one of these, previously asymptomatic, develops pyrexia, weakness, malaise and sweating from an attack of influenza, he will be found to have these high brucella antibodies. In the hands of the uncritical or inexperienced the serological findings may be interpreted as evidence of active brucellosis.

Much more information is required about the use of these tests in clinical practice. Kerr *et al.* (1966*b*) are right in emphasizing the importance of negative

results in excluding a diagnosis of brucellosis. It is not so easy to know how to interpret positive results in order to confirm the diagnosis. Kerr *et al.* (1966*b*) may be right in their statement that the CF test detects microglobulins which are directly associated with the activity of infection and that these microglobulins are present in both the chronic and acute stages of the illness in titres of 16 or greater, but this present study would suggest that such a level of positivity will not help the physician faced with a patient who is occupationally exposed to brucellosis. Titres of 160 and 128 or greater for the AHG and CF tests respectively in a symptomatic patient may be a more realistic and useful guide in the absence of culture of the organism, but they are not diagnostic and the final decision must still be taken by the physician after careful consideration of both clinical and epidemiological factors.

I wish to thank Professor O. L. Wade and Professor P. C. Elmes for their encouragement and helpful advice; Dr W. R. Kerr and Dr W. J. McCaughey of the Veterinary Research Laboratories, Belfast, for carrying out the laboratory procedures; Dr J. D. Merrett for advice about statistics; and Dr J. H. Connolly for his co-operation and assistance.

This work was supported in part by research awards from the Queen's University of Belfast and the Northern Ireland Hospitals Authority.

REFERENCES

Bartram, H. G., Bothwell, P. W., Jebb, W. H. H., McDiarmid, A. & Preston, A. E. (1963). *Brucella abortus* agglutinins in the sera of pregnant women and blood donors. *British Journal of Preventive and Social Medicine* **17**, 95.

Bradstreet, C. M. P. & Taylor, C. E. D. (1962). Technique of complement fixation test applicable to the diagnosis of virus diseases. *Monthly Bulletin of the Ministry of Health and Public Health Laboratory Service* **21**, 96.

Dalrymple-Champneys, W. (1929). Undulant fever with special reference to animal sources of infection and the possibility of its prevalence in England and Wales. *Reports on Public Health and Medical Subjects, Ministry of Health*. no. 56.

Dalrymple-Champneys, W. (1960). *Brucella Infection and Undulant Fever in Man*. Oxford University Press.

Evans, A. C., Robinson, F. H. & Baumgartner, L. (1938). Studies on chronic brucellosis. IV. An evaluation of the diagnostic laboratory tests. *Public Health Reports, Washington* **53**, 1507.

Henderson, R. J. (1967). Brucellosis in the dairy-farming community and allied workers of Worcestershire. *Lancet* ii, 353.

Heremans, J. F., Vaerman, J. P. & Vaerman, C. (1963). Studies on the immune globulins of human serum. II. A study of the distribution of anti-brucella and anti-diphtheria antibody activities among gamma-SS, gamma-IM and gamma-IA globulin fractions. *Journal of Immunology* **91**, 11.

Joint FAO/WHO Expert Committee on Brucellosis (1964). 4th Report. *Technical Report Series, World Health Organization*, no. 289.

Kerr, W. R., Coghlan, J. D., Payne, D. J. H. & Robertson, L. (1966*a*). Chronic brucellosis in the practising veterinary surgeon. *Veterinary Record* **79**, 602.

Kerr, W. R., Coghlan, J. D., Payne, D. J. H. & Robertson, L. (1966*b*). The laboratory diagnosis of chronic brucellosis. *Lancet* ii, 1181.

Lancet, Annotation (1966). Brucellosis among veterinary surgeons. *Lancet* ii, 952.

McDevitt, D. G. (1968). Studies in Human Brucellosis. M.D. Thesis, Queen's University, Belfast.

McDevitt, D. G. & McCaughey, W. J. (1969). Brucellosis in Northern Ireland—a serological survey. *Journal of Hygiene* **67**, 409.

Macdonald, A. & Elmslie, W. H. (1967). Serological investigations in suspected brucellosis. *Lancet* i, 380.

Reddin, J. L., Anderson, R. K., Jenness, R. & Spink, W. W. (1965). Significance of 7S and macroglobulin brucella agglutinins in human brucellosis. *New England Journal of Medicine* **272**, 1263.

Spink, W. W. (1951). What is chronic brucellosis? *Annals of Internal Medicine* **35**, 358.

Spink, W. W. (1956). *The Nature of Brucellosis.* Minneapolis: University of Minnesota Press.

Spink, W. W., McCullough, N. B., Hutchings, L. M. & Mingle, C. K. (1952). Diagnostic criteria for human brucellosis. *Journal of the American Medical Association* **149**, 805.

Wilson, M. M. & Merrifield, F. V. O. (1951). The anti-globulin (Coombs) test in brucellosis. *Lancet* ii, 913.

J. Hyg., Camb. (1970), **68**, 189
Printed in Great Britain

A survey on the occurrence of *Vibrio parahaemolyticus* on fish and shellfish, marketed in The Netherlands

By E. H. KAMPELMACHER, D. A. A. MOSSEL, L. M. van NOORLE JANSEN and H. VINCENTIE

National Institute of Public Health, Utrecht, The Netherlands, and Central Institute for Nutrition & Food Research T.N.O., Zeist, The Netherlands

(*Received* 6 *November* 1969)

SUMMARY

A survey was carried out on the occurrence of *Vibrio parahaemolyticus* on fish and shellfish, as sold in The Netherlands.

The optimal mode of detection of this bacterium appeared to be: (i) enrichment of swabs taken from the surface and the gills in freshly prepared meat broth with 5% NaCl; (ii) streaking onto Teepol bromothymol blue agar (BTB) and taurocholate bromothymol blue sucrose agar (TCBS); (iii) confirmation of suspect colonies by testing for mode of growth in butts/slants of a Kligler type glucose sucrose iron thiosulphate agar, formation of indole in 2% NaCl 2% trypticase water, anaerobic utilization of starch in the presence of 5% NaCl and oxidase reaction according to Kovacs (1956).

A total of 407 samples, stemming from 17 types of fish and shellfish, taken at three fish shops, was examined by this technique. Only one specimen, i.e. a haddock, was found to contain *V. parahaemolyticus*. This contamination rate of approximately 0·3% correlates well with data found earlier for fish landed in Northern Germany.

INTRODUCTION

Epidemiological investigations carried out in Japan have demonstrated that the halotrophic Vibrio species *V. parahaemolyticus* can cause outbreaks of infectious enteritis when absorbed with food in great numbers (Sakazaki, Iwanami & Fukumi, 1963; Zen Yoji *et al.* 1965; Sakazaki *et al.* 1968). Such vibrios have their main habitat in estuarine waters, fish and shellfish. Outside Japan, these bacteria have been encountered on fish and shellfish landed in the United States (Ward, 1968; Krantz, Colwell & Lovelace, 1969) and in Northern Germany (Nakanishi, Leistner, Hechelmann & Baumgart, 1968).

This evidence prompted a search for *V. parahaemolyticus* in fish and shellfish marketed in The Netherlands. Before starting the survey proper, systematic attempts were made to develop a method of detection of these vibrios in such products, which would combine the convenience required for survey work with reasonable sensitivity.

DEVELOPMENT OF A METHOD OF DETECTION

As *V. parahaemolyticus* is a non-psychrotrophic mesophilic organism (Temmyo, 1966), it may be assumed that on fresh, chilled fish and shellfish only relatively low numbers of vibrios will occur. This requires that a search for these bacteria in fish, etc. is made by a suitable enrichment technique, followed by subculturing for isolation on an appropriate solid medium.

The value of various combinations of media for this purpose was assessed.

Methods

Strains

Eight pure cultures of *V. parahaemolyticus* were used. These had been made available by Dr R. Sakazaki, National Institute of Health, Tokyo, Japan.

In addition pure cultures of various other food bacteria, stemming from the Institutes' collections, were used.

Enrichment media

The following media were used: (i) freshly prepared meat broth with 5% NaCl; (ii) the Japanese selective enrichment medium, containing 2% NaCl and 60 μg./ml. colistin methane sulphonate (Sakazaki *et al.* 1963); (iii) the same as (ii), but with 10 μg./ml. of the allied antibiotic polymyxin B sulphate instead of colistin, since we had found earlier that this adequately suppresses many Gram-negative rod-shaped bacteria (Mossel, 1959); (iv) the same as (iii), but with, in addition, 5 μg./ml. tylosin added, to suppress particularly bacilli and catalase positive cocci (Greenberg & Silliker, 1962*a*, *b*); (v) the enrichment medium, currently used for the detection of *V. cholerae* in stools, containing peptone, 5 g.; NaCl, 5 g.; glucose, 5 g.; teepol (Shell Netherlands), 1 ml.; methyl violet, 2 mg.; water, 1 l.; pH = 8·5 (Sakazaki, 1965). In order to attain maximal inhibition of the *Pseudomonas–Acinetobacter* association that predominates on chilled fish and shellfish, enrichment cultures were tentatively also made under anaerobic conditions, attained by covering the broth surface with sterile beef tallow.

Isolation media

In the initial work the two original Japanese isolation media were used. These are: (i) BTB-agar (Akiyama *et al.* 1963), containing meat extract, 3 g.; peptone, 10 g.; sucrose, 15 g.; NaCl, 20 g.; teepol (Shell Netherlands), 1 ml.; bromothymol blue, 80 mg.; agar, 15 g.; water, 1 l.; final pH = 7·8; (ii) TCBS-agar (Kobayashi, Enomoto, Sakazaki & Kuwabara, 1963), of the following composition: yeast extract powder, 5 g.; meat extract paste, 5 g.; peptone, 10 g.; NaCl, 10 g.; $Na_2S_2O_3.5H_2O$, 10 g.; dried ox bile, 5 g.; sodium taurocholate, 3 g.; sodium citrate, 10 g.; ferric citrate, 1 g.; sucrose, 15 g.; bromothymol blue, 40 mg.; thymol blue, 40 mg.; agar, 15 g.; water 1 l.; final pH = 9.2.

In tentative studies with mixtures of pure cultures of *V. parahaemolyticus* and some other types of bacteria of common occurrence on fish the selectivity of these

media was found rather disappointing. Hence we attempted to modify them so as to inhibit particularly Gram-positive organisms, *Pseudomonadaceae* and some *Enterobacteriaceae*. This was tried by the addition to BTB-agar of 5% NaCl, 5 μg./ml. tylosin and 2 μg./ml. polymyxin B sulphate as inhibitors. After such an agar, called PTSS (Polymyxin Tylosin Salt Sucrose), had been found sufficiently selective, its productivity was tested, using the eight pure cultures of *V. parahaemolyticus*, referred to earlier. Although this appeared to be rather low, viz. 10^{-3}–$< 10^{-6}$ of colonies growing on blood agar developing on this agar medium, we yet decided to assess its value in comparison with the other two media.

Identification procedures

The cardinal taxonomic properties of *V. parahaemolyticus* are the following: catalase +, oxidase +, glucose attacked by a fermentative pathway, sucrose −, H_2S from cysteine and thiosulphate −, indole +, nitrate +, urea −, gelatin +, arabinose +, growthin the presence of 10% Na − Cl(Sakazaki *et al.* 1968). In addition Baross & Liston (1968) observed that *V. parahaemolyticus* utilizes starch under anaerobic conditions in the presence of 5% NaCl. We have investigated whether this criterion could be used as a rapid screening test for the confirmation of suspect colonies obtained on BTB-and/or TCBS-agar.

Various fresh isolates of the genera *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Aeromonas*, *Klebsiella* and *Staphylococcus* were stabbed to the bottom of tubes, containing a 10 cm. butt of freshly steamed agar of the following composition: trypticase, 10 g.; yeast extract, 3 g.; NaCl, 50 g.; soluble starch, 10 g.; bromothymol blue, 80 mg.; agar, 15 g.; water 1 l.; pH = 7·2. After 24–40 hr. incubation at 37° C. growth and change of the colour of the indicator to yellow in the lowest part of the tube were checked. We had established earlier that the latter phenomenon indicates very reliably whether an isolate is capable of anaerobic dissimilation of carbohydrates (Mossel & Martin, 1961). The results of these tests were that, although some of the tested organisms, e.g. staphylococci and some *Enterobacteriaceae*, would tolerate the NaCl concentration used, none of these would attack starch; whereas the generally amylase positive aeromonadaceae (Schubert, 1967) would not grow in the presence of 5% NaCl. Attempts to inhibit the former bacteria by increasing the NaCl content to the required level of 8% failed, because at this reduced a_w-value the anaerobic attack on starch by *V. parahaemolyticus* became less consistent.

In the survey therefore a freshly steamed deep tube of 5% NaCl starch agar was used in the identification, together with a Kligler type sucrose glucose thiosulphate iron agar in butt/slant form, a tube of 2% trypticase 2% NaCl broth for testing indole formation and the oxidase reaction by the method of Kovacs (1956).

Results

Enrichment media

Amongst the five enrichment media described under Methods, the alkaline peptone teepol methyl violet medium lacked sufficient selectivity. The three other

Table 1. *A comparison of the results with various techniques for the detection of* V. parahaemolyticus *added to fish swabs*

Strains of *V. parahaemolyticus* used	Approx. numbers of cells initially present per 10 ml. enrichment fluid	Enrichment in polymyxin 2 % NaCl meat broth: Aerobic incubation 20 hr. at 37° C.			Enrichment in polymyxin 2 % NaCl meat broth: Anaerobic incubation 20 hr. at 37° C.			Enrichment in 5 % NaCl meat broth: Aerobic incubation 20 hr. at 37° C.			Enrichment in 5 % NaCl meat broth: Anaerobic incubation 20 hr. at 37° C.		
		BTB	TCBS	PTSS	BTB	TCBS	PTSS	BTB	TCBS	PTSS	BTB	TCBS	PTSS
V_1	*ca.* 400	+ +†	+	+	+ +	−	−	+ + +	+ + +	+ +	+ + +	+ + +	+ +
	40	+ +	−	−	+ +	+	−	+ + +	+ + +	+ +	+ + +	+ + +	+ +
V_2	*ca.* 660	+ +	+ +	+ +	+ +	+ +	+	+ + +	+ + +	+ +	+ + +	+ + +	+ +
	66	+ +	+ +	+ +	−	+	−	+ +	+ +	+	+ +	+ + +	+ +
V_4	*ca.* 360	+ +	−	−	+ +	+	+	+ +	+ +	+ +	+ +	+	+ +
	36	+ +	−	−	+ +	+	+	+ +	+ +	+ +	+ +	+ + +	+ +
V_5	*ca.* 480	+ +	+ + +	+ +	+ +	+ +	−	+ +	+ + +	+	+ +	+ +	+
	48	+ +	+ +	−	+	−	−	+ + +	+ + +	+ +	+ +	+ +	+
V_6	*ca.* 300	+ +	+ +	+ +	+ +	+ +	+	+	+ +	+	+	+ +	+
	30	+ +	+	+	−	+	−	+ +	+ +	+	+	+ +	+
V_8	*ca.* 310	+ + +	+ + +	+	+ +	+ +	−	+ + +	+ +	+	+ + +	+ +	+
	31	+ + +	+ + +	+	+ +	+ +	−	+ +	+ +	+	+ + +	+ +	+
Number of missed isolations in the case of lowest inoculum		0	2	3	2	1	5	0	0	0	0	0	0
Growth index†		26	19	12	19	15	4	28	29	18	27	28	18
Control: non-inoculated enrichment fluid with fish swabs		—	—	—	—	—	—	—	—	—	—	—	—

† Growth scale used for colonies of *V. parahaemolyticus*.
\+ Scarce growth (< 30 col.).
\+ + Growth (30–300 col.).
\+ + + Luxuriant growth (> 300 col.).
− No growth

BTB Bromothymol blue teepol sucrose salt agar (pH 7·8).
TCBS Taurocholate citrate bromothymol blue sucrose salt iron agar (pH 9·2).
PTSS Polymyxin tylosin sucrose salt agar.

selective media used for enrichment gave excellent results with pure cultures of *V. parahaemolyticus*, but when these same strains were added to fish swabs at about 100 organisms per swab all these three media showed a lower recovery rate than the antibiotic-free 5% NaCl meat broth, which was therefore chosen for use in the survey.

Isolation media

The relative values of the three solid media for isolating *V. parahaemolyticus* from two different enrichment media were determined by the following procedure.

Tubes of the enrichment media were inoculated with two different concentrations of the *Vibrio* suspensions giving about 400 and 40 organisms per 10 ml. of medium respectively. Swabs which had been infected with normal fish flora by rubbing them against fresh Dutch sole were added to these inoculated tubes, which were thereupon incubated aerobically and anaerobically for 20 hr. at 37°C. Each tube was then subcultured on plates of the three different isolation media, which were incubated for 24–48 hr. at 37°C.

The results shown in Table 1, particularly the numbers of missed isolations shown in line 13 of the table, demonstrate clearly that PTSS-agar is too inhibitory for this purpose. Although there were no missed positives when PTSS-agar was used in conjunction with 5% NaCl meat broth, the number of colonies on the plates was always low, with a 'growth index' of 18 against 27–29 for the other two media. It should be stressed, however, that this does not detract from the value of the PTSS medium for other purposes, such as replication of master plates of food dilutions for tentative grouping of bacteria encountered in foods (Mossel, 1969).

Anaerobic incubation of polymyxin broth gave a low recovery compared with aerobic, but with 5% NaCl broth the two methods were equally good.

Sensitivity of the method finally adopted

As a result of these preliminary tests the best method for the isolation of *V. parahaemolyticus* from fish appeared to be by enrichment in 5% NaCl meat broth for 20 hr. at 37°C. followed by plating on BTB-agar or TCBS-agar, or both, with incubation for 20 hr. at 37°C.

To assess the sensitivity of this method, inocula of about 100 cells of each of the *Vibrio* strains studied were added to the enrichment medium, simultaneously with 10^4–10^5 viable cells of a mixed inoculum of *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Arizona* sp., *Escherichia coli* and *Enterobacter aerogenes*. By the use of the selected procedure the *Vibrio* strains could invariably be isolated. Under more severe conditions, i.e. in the presence of large numbers of the *Pseudomonas–Acinetobacter* association of fish, the procedure appeared to be less productive: when artificially inoculated with 1–10 cells of *V. parahaemolyticus* these organisms were successfully recovered from 13 out of 26 swab cultures; when inoculated with 10–10^2 cells 40 out of 48 of such cultures were positive.

THE SURVEY PROPER

Principles

Any *V. parahaemolyticus* present on fish may be expected to occur on the surface, because these bacteria stem primarily from estuarine waters. Therefore it seemed justified to sample fish by a non-destructive surface technique. Plugs of cotton wool, previously found to preserve *V. parahaemolyticus* adequately (Mossel, Kampelmacher & van Noorle Jansen, 1968), were used for this purpose.

The entire body surfaces of the fish and shellfish were streaked intensively. In fish particularly the gills were included in this swabbing. The swabs were immediately transferred to tubes of 5% NaCl meat broth. Depending on the size of the fish, two to three swabs were used per object.

In this survey a check on the functioning of the procedure was built in. For this purpose two swabs, artificially inoculated with 10–100 *V. parahaemolyticus*, were tested in parallel with the fish samples. *V. parahaemolyticus* was recovered from those controls in all instances, except one, which was carried out along with a series of tests done on an extremely warm day, with an ambient temperature over 30°C.

Material, results and discussion

In the period June–September 1968 and 1969 a total of 407 samples were examined. The samples originated from the North Sea and were taken in three different fish shops in the City of Utrecht. The types of fish and shellfish examined in this survey and their numbers are summarized in Table 2.

From one sample only, *viz.* a haddock, *V. parahaemolyticus* was isolated. The

Table 2. *Review of types of fish and shellfish samples examined for* V. parahaemolyticus

Type	Number of samples examined
Bass	9
Cod	62
Codling	2
Garfish	6
Green cod	3
Gurnard	20
Haddock	49
Herring	2
Mackerel	28
Mullet	15
Plaice	42
Sole	42
Turbot	19
Whiting	24
Lobster	26
Shrimp	33
Cuttle fish	25
Total	407

contamination rate was therefore 0·25%. Nakanishi, Leistner & Baumgart (1967) and Nakanishi *et al.* (1968) had earlier found no positives in fish landed from the North Sea, and 3–31%, dependent on the month in which sampling was carried out, in fish landed from the Baltic Sea. Our results lie between these limits.

It appears from this survey that *V. parahaemolyticus* is not frequently encountered on fisheries products as sold in The Netherlands. Most of the commodities studied in this survey are stored in chilled, unsalted form. In addition, they are always eaten after cooking or frying. These two circumstances respectively, do not favour the growth of *V. parahaemolyticus* and will eliminate any organism of this type that might develop. Together with the low degree of contamination found in fish and shellfish, these circumstances make it rather unlikely that *V. parahaemolyticus* plays an important role in gastro-enteritis in The Netherlands.

The authors are highly indebted to Dr R. Sakazaki for sending the *V. parahaemolyticus* strains used in the preliminary work described in this publication, and especially to Mr M. Wijnschenk and Mrs A. Voest, fishmongers, and 'Marijn', a firm dealing in fish, for their excellent co-operation in this investigation. They are also very grateful to Mr G. B. Engels, Mrs E. P. van Leusden-Meeuwsen and Miss S. A. Kalksma, who have carried out sampling in the various shops.

REFERENCES

Akiyama, S., Takizawa, K., Ichinohe, M., Enomoto, S., Kobayashi, S. & Sakazaki, R. (1963) Application of teepol to the isolation medium for the enteropathogenic halophilic vibrios. *Japanese Journal of Bacteriology* **18**, 255.

Baross, J. & Liston, J. (1968). Isolation of *Vibrio parahaemolyticus* from the Northwest Pacific. *Nature London* **217**, 1263–4.

Greenberg, R. A. & Silliker, J. H. (1962). The effect of tylosin on coagulase positive staphylococci in food products. *Journal of Food Science* **27**, 60–3.

Greenberg, R. A. & Silliker, J. H. (1962). The action of tylosin on sporeforming bacteria. *Journal of Food Science* **27**, 64–8.

Kobayashi, T., Enomoto, S., Sakazaki, R. & Kuwabara, S. (1963). A new selective isolation medium for pathogenic vibrios: TCBS-agar. *Japanese Journal of Bacteriology* **18**, 387–91.

Kovacs, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature London* **178**, 703.

Krantz, G. E., Colwell, R. R. & Lovelace, E. (1969). *Vibrio parahaemolyticus* from the blue crab *Callinectes sapidus* in Chesapeake bay. *Science* **164**, 1286–7.

Mossel, D. A. A. (1959). Enumeration of sulphite-reducing clostridia occurring in foods. *Journal of the Science of Food & Agriculture* **10**, 662–9.

Mossel, D. A. A. (1969). Microbiological quality control in the food industry. *Journal of Milk & Food Technology* **32**, 155–71.

Mossel, D. A. A., Kampelmacher, E. H. & van Noorle Jansen, L. M. (1968). The optimal mode of transport for swabs obtained from surfaces examined for organisms causing food-borne disease. *Journal of Hygiene* **66**, 59–66.

Mossel, D. A. A. & Martin, G. (1961). Milieu simplifié permettant l'étude des divers modes d'action des bactéries sur les hydrates de carbone. *Annales de l'Institut Pasteur de Lille* **12**, 225–6.

Nakanishi, H., Leistner, L. & Baumgart, J. (1967). Nachweis von *Vibrio alginolyticus* bei Seefischen in Deutschland. *Archiv für Lebensmittelhygiene* **18**, 201–2.

Nakanishi, H., Leistner, L., Hechelmann, H. & Baumgart, J. (1968). Weitere Untersuchungen über das Vorkommen von *Vibrio parahaemolyticus* und *Vibrio alginolyticus* bei Seefischen in Deutschland, *Archiv. für Lebensmittelhygiene* **19**, 49–53.

SAKAZAKI, R. (1965). *Vibrio parahaemolyticus*. A non-choleragenic enteropathogenic Vibrio. *Proceedings of the Cholera Research Symposium. Honolulu, Hawaii*, 30–34 (Dept. of Health, Education and Welfare, Public Health Service, Washington).

SAKAZAKI, R., IWANAMI, S. & FUKUMI, H. (1963). Studies on the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus*. I. Morphological, cultural and biochemical properties and its taxonomic position. *Japanese Journal of Medical Science & Biology* **16**, 161–88.

SAKAZAKI, R., TAMURA, K., KATO, T., OBARA, Y., YAMAI, S. & HOBO, K. (1968). Studies on the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus*. III. Enteropathogenicity. *Japanese Journal of Medical Science & Biology* **21**, 325–31.

SCHUBERT, R. H. W. (1967). Das Vorkommen der Aeromonaden in oberirdischen Gewässern. *Archiv für Hygiene und Bakteriologie (Berl.)* **150**, 688–708.

TEMMYO, R. (1966). Studies on the prevention of outbreaks of food poisoning caused by *Vibrio parahaemolyticus*. *Bulletin of Tokyo Medical & Dental University* **13**, 489–510.

WARD, B. Q. (1968). Isolations of organisms related to *Vibrio parahaemolyticus* from American estuarine sediments. *Applied Microbiology* **16**, 543–6.

ZEN-YOJI, H., SAKAI, S., TERAYAMA, T., KUDO, Y., ITO, T., BENOKI, M. & NAGASAKI, M. (1965). Epidemiology, enteropathogenicity and classification of *Vibrio parahaemolyticus*. *Journal of Infectious Diseases* **115**, 436–44.

J. Hyg., Camb. (1970), **68**, 197
Printed in Great Britain

Inactivated smallpox vaccine. A comparison of inactivation methods

By G. S. TURNER, E. J. SQUIRES and H. G. S. MURRAY
The Lister Institute of Preventive Medicine, Elstree, Hertfordshire

(*Received* 6 *November* 1969)

SUMMARY

Vaccines were prepared from a single pool of high-titred vaccinia virus and inactivated by six methods, namely heat, formalin, hydroxylamine, β-propiolactone, ultraviolet irradiation, and visible light and methylene blue. Large doses of the vaccines were required to protect mice against intracerebral challenge. Differences in protection were not attributable to the method of their inactivation. The vaccines also induced similar degrees of skin immunity in rabbits which showed no severe dermal reactions when challenged with either homologous killed vaccine or live virus. The virus-neutralizing, haemagglutinin-inhibiting and complement fixing antibody responses to the vaccines differed; heat-inactivation preserved these antigens least well and β-propiolactone apparently the best. In both rabbits and mice there was little association between the different antibody responses to each vaccine or between the degrees of antibody response and the protection they induced. The relation of these findings to pox-virus immunity and the use of inactivated smallpox vaccine in man is discussed.

INTRODUCTION

There have been many attempts to produce an inactivated smallpox vaccine that would obviate the inherent hazards of conventional Jennerian vaccination. However, differences in preparation and in the assessment of their efficacy as antigens, either in man or animals, have produced many conflicting results (Janson, 1891; Parker & Rivers, 1936; Donally & Weil, 1940; Weil & Gall, 1940; Collier, McClean & Vallet, 1955; Herrlich, 1959; Beunders, Driessen & van den Hoek, 1960; Mahnel, 1960; Amies, 1961; von Epp, 1961; Lindenman & Buser, 1962; Kaplan, McClean & Vallet, 1962; Ramano Rao, 1962; Kaplan, Benson & Butler, 1965; Turner & Kaplan, 1965; McNeill, 1965; Madeley, 1968). Vaccines prepared from virus grown on different hosts have varied in antigen content, some were incompletely inactivated, and others may have lost immunogenicity as the result of the chemical or physical treatment used for inactivation. In the present study six commonly used methods of inactivation were used to make vaccines from a single batch of high titred virus. Immunogenicity was assessed by antibody response and skin immunity in rabbits, and by protection tests in mice. Immunization schedules and dosage were limited to those thought to be practicable when translated for use in man.

MATERIALS AND METHODS

Viruses

The Lister Institute strain of vaccinia virus was used for the preparation of vaccines. It was extracted from sheep dermal pulp in 0·015M phosphate buffered saline at pH 7·0, purified by treatment with 'Arcton 113' (trifluorotrichloroethane I.C.I.) and differential centrifugation. A stock suspension containing 10^9 to 10^{10} pock-forming units (pk.f.u.)/ml. was stored at $-160°$ C. in 20 ml. volumes. Virus for the dermal challenge of rabbits was derived from chick chorioallantoic membranes (CAM) infected with the same strain. It was prepared and stored similarly, contained 3×10^9 pk.f.u./ml. and was bacteriologically sterile. The neurotropic strain of vaccinia virus (W.R.) used for intracerebral (i.c.) challenge of mice was a freeze-dried preparation of mouse brain. It was stored at 0–4° C.; when reconstituted and titrated in 16–20 g. mice it contained 10^4 LD50/0·02 ml.

Virus assay

0·1 ml. volumes of suitable dilutions of samples were inoculated on the CAM of 12-day chick embryos. Virus was estimated from the mean pock counts of 4–6 membranes after 48 hr. incubation. Titres are expressed in pk.f.u./ml.

Inactivation procedures

Dye and visible light. Virus suspensions were illuminated with a Philips Photolita bulb at 50 ft.c. intensity for 45 min. in the presence of methylene blue (3×10^{-5}M) (Turner & Kaplan, 1965).

Ultraviolet light. Virus suspensions were exposed for 30 min. in open Petri dishes 30 cm. distant from a UV tube (Philips 30 watt).

Heat. Virus sealed in suitable containers was completely immersed in a water bath at 60° C. for 40 min.

Formalin. Virus was stirred for 24 hr. at 18–20° C. in a dark container in the presence of formaldehyde (0·03M) (B.D.H.A.R.) and glycine (0·04M) (Gard, 1957).

Hydroxylamine. Hydroxylamine hydrochloride (Hopkins & Williams A.R.) was freshly prepared as a 2M solution. The free acid was neutralized with NaOH before dilution to a final concentration of 0·1M in the virus mixture. Inactivation was erratic and was only complete after long exposure (60–72 hr.) at 18–20° C. in the dark.

β-propiolactone (*B.P.L.*). Concentrations ranging between 1/250 and 1/500 were used, the mixture being held either at 0–4° C. or mechanically stirred for 24 hr. at 18° C. Free acid produced during the hydrolysis of the inactivator was neutralized with a few drops of Na_2CO_3 (0·5M). The inactivation of most of the virus was rapid, but there were often traces of viable virus left that required more agent to ensure complete inactivation.

Preparation of vaccines

Stock virus was thawed, diluted fivefold, given approximately 30 sec. ultrasonic treatment to disperse aggregates and mixed in 20 ml. lots with an equal volume of

inactivator. Each mixture was exposed for the required time and temperature to effect just complete inactivation. The virus was sedimented from the mixture at 20,000 *g* for 30 min. and resuspended in the original volume of 0·015 M phosphate buffered saline (pH 7·0). The suspensions were again treated ultrasonically, and tested for inactivation. Stock virus submitted to similar procedures in the absence of inactivator controlled the efficacy of virus recovery, and provided virus for comparison with inactivated virus. All the inactivated vaccines contained virus equivalent to $10^{8.7}$ to 10^{9} pk.f.u./ml. They were stored at $-160°$ C. and thawed for immediate use, all unused material being discarded.

Tests for inactivation of vaccines

All the killed vaccines were submitted to the series of rigorous tests described by Madeley (1968), to determine whether viable or reactivable virus was still present. These were briefly

(1) Two successive chorioallantoic passages in eggs to detect virus survival or multiplicity reactivation.

(2) Inoculation on the chorioallantoic membrane in the presence of a known quantity of live virus to detect interference by large quantities of dead virus or the reactivation of dead by live virus.

(3) Intradermal inoculation into the shaved flanks of rabbits to detect dermal toxicity or viable virus.

In our hands, however, the intradermal inoculation of rabbits proved to be an unreliable test for complete inactivation. Some samples negative in rabbits yielded viable virus after two passages in eggs. A total volume of 1·0–2·0 ml. of each vaccine was tested. Vaccines were not used if any evidence of viable virus was obtained.

Immunogenicity of the vaccines

Mouse protection

Subcutaneous injection of large doses of most strains of vaccinia virus produces neither lesions nor illness in adult mice although transient infection occurs with live virus (Rosenau & Andervont, 1931; Briody, 1959; Turner, 1967). Male T.O. strain mice (Scientific Animal Service, Elstree) were used at an initial weight of 11–14 g. The methods of immunization and challenge are similar to those used in the NIH potency test for rabies vaccines (Seligmann, 1966). Three serial tenfold dilutions were prepared from each vaccine in dilute (0·015 M) phosphate buffered saline (pH 7·0). Each dilution of vaccine was used to immunize a group of 10–20 mice; a similar group received undiluted vaccine. Two subcutaneous injections of 0·5 ml. were given at intervals of 1 week. Similar groups of mice were immunized with dilutions of live virus. One week after the second dose 5–10 mice from each group receiving the undiluted vaccine were killed and bled for antibody assay. The remaining mice were challenged intracerebrally with an estimated 5–50 LD50 of the neurotropic vaccinia strain W.R., which was titrated with each test in normal mice of the same batch. Mice dying within 48 hr. of challenge were discarded and the remainder were observed for 21 days. The 50% effective dose

(ED50) of each vaccine was calculated as the number of pock-forming units of virus, live or inactive, protecting 50% of the mice against the lethal challenge. Each vaccine was tested twice in this way.

Interferon in mice

Thirty mice were immunized with live virus; 10 were killed, bled and their brains removed. The remainder were challenged as above, and 24 hr. later blood and brains were taken from 10 of them. The 10 remaining mice, observed for 21 days, all survived. Control mice challenged in the same way were similarly sampled. Interferon was assayed in 10% (w/v) brain homogenates by the methods of Gifford (1963) and Subrahmanyan & Mims (1966). Virus-neutralizing (VN) antibody in sera and brain homogenates was estimated (see below).

Immunization of rabbits

New Zealand Red male rabbits weighing approximately 2 kg. were selected for skin areas free from pigmentation, hyperaemia and abnormal rates of hair growth. Each undiluted vaccine was tested in four rabbits in two intramuscular 1·0 ml. doses given 1 week apart. Four uninoculated rabbits served as controls. Four rabbits were vaccinated with live virus and housed in an area remote from those receiving either inactivated or no vaccine. The animals were bled before and 7 days after immunization and again 16 days after challenge.

Skin sensitivity

McNeill (1966) suggests that exaggerated skin reactivity may be induced by virus treated with particular inactivators. Accordingly two rabbits from each group were tested with 0·1 ml. intradermal doses of serial tenfold dilutions of killed virus 1 week after their second immunizing dose. In each case the immunizing and test material had been inactivated by the same method. To avoid any reactions due to sensitization by host components, the test material was made from virus grown in CAM and not in sheep. Two rabbits immunized with live virus were at the same time tested with a 1/10 dilution of all the variously inactivated preparations. Saline was used as a control. Photographic records as well as visual estimates of any dermal reactions were made after a few hours, and daily for 1 week. The animals were bled 16 days after the skin test, and challenged dermally with live virus.

Dermal challenge

One week after their second immunizing dose, 0·1 ml. of serial fivefold dilutions of live, bacteriologically sterile, CAM virus was applied to the scarified skin of the two remaining rabbits in each group. The animals were inspected daily, and the degree of skin resistance determined from readings on the fifth day, as in potency assays of smallpox vaccine. Photographic records as well as visual estimates of the lesions were made during the 14 days following challenge.

Antibody assays

Serum samples were inactivated at 56° C. for 45 min. and stored at −15° C.

Virus neutralizing antibody

This antibody was assayed as described by Turner & Kaplan (1965). Neutralizing potencies are expressed in international units (i.u.) calculated by reference to the potency of the international standard smallpox antiserum titrated with each assay. Each sample was assayed at least twice.

Haemagglutination inhibition

Vaccinia haemagglutinin was prepared from chorioallantoic membranes infected with Lister strain virus. They were extracted in saline and gross particles sedimented by low speed centrifugation. Haemagglutinin in the supernatant fluid was estimated with sensitive fowl cells. It was stored without further purification at −160° C. Dilutions of the sera were tested against eight agglutinating doses of haemagglutinin with 0·5 % fowl cells as indicator; unit volumes were 0·2 ml., and serum-saline was used as diluent (McCarthy & Helbert, 1960).

Complement fixation

Soluble antigen was prepared from a 10 % (w/v) suspension of sheep dermal pulp in phosphate buffered saline (pH 7·0). It was centrifuged at 20,000 *g* for 1 hr., and the supernatant fluid filtered through a millipore filter (pore size 0·22 μ). The filtrate was distributed in convenient volumes and stored at −15° C. When titrated against a hyperimmune sheep antivaccinial serum it had a titre of > 1/640 and was not anti-complementary when used in tests at a 1/20 dilution. The testing techniques and other reagents have been described (Madeley, 1968).

Gel precipitin tests

Undiluted 'soluble antigen' prepared for the complement fixation tests above was tested against undiluted and suitable dilutions of sera by gel diffusion in agar plates (Rondle & Dumbell, 1962). Hyperimmune sheep, normal rabbit and mouse serum were used as controls.

RESULTS

Immunogenicity in mice

The inactivated vaccines protected the mice only with doses 1000- to 10,000-fold larger than the protective dose of live virus. The dose response to all vaccines was linear and with one exception the slopes were parallel (Fig. 1). The small protective dose of live virus probably indicates the amount of Lister strain virus which establishes infection and viral multiplication in mice. The differences between the inactivated vaccines are not statistically significant.

Antibody response in mice

The antibody responses of mice are unrelated to protection (Table 1). No significant amounts of complement-fixing (CF) antibody were produced by any vaccine; as an index of an immune response to vaccinia in mice it appears to have little value. Neither was haemagglutinin-inhibiting (HI) antibody induced by most of the vaccines, it appeared irregularly after live virus and BPL-inactivated vaccine.

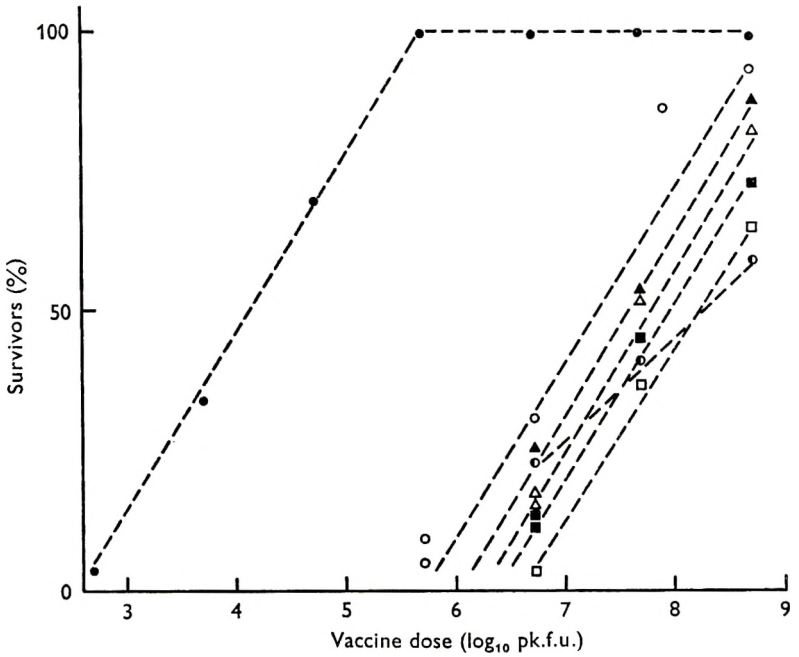

Fig. 1. Protection of mice with live and inactivated vaccines against intracerebral challenge (Av. 19 LD50). ●, Live vaccine; ○, vaccine inactivated by heat; ▲, formalin; △, β-propiolactone; ■, hydroxylamine; □, ultraviolet irradiation; ◑, photoinactivation.

Table 1. *Response of mice to live and inactivated vaccines*

Method of inactivation of vaccine	Mean ED50 (log_{10} pk.f.u.)	Antibodies* at time of challenge		
		VN	HI	CF
Heat	7·4	0·3	< 5·0	< 20
β-Propiolactone	7·5	25	40	25
Formalin	7·7	7·2	< 5·0	< 20
Photodynamic	8·5	0·9	< 5·0	< 20
Ultraviolet	7·7	16·0	< 5·0	NT
Hydroxylamine	8·1	0·9	< 5·0	25
None (live virus)	3·9	1·2 to 90	< 5 to 40	< 20
No vaccine	—	< 0·05	< 5	< 20

* VN = Virus neutralization (International Units).
HI = Haemagglutinin inhibition (reciprocal titre).
CF = Complement fixation (reciprocal titre).
NT = Not tested.

Although the inactivated vaccines were equally protective, that inactivated by BPL induced comparatively high titres of virus-neutralizing (VN) and HI antibody, suggesting either that BPL preserved these antigens or that traces of live virus escaped detection in this vaccine (Kaplan, 1962). The ED50 of live virus makes this unlikely (Table 1) although vaccine treated more vigorously with this reagent gave an almost negative response.

Most of the vaccines induced VN antibodies, but in extremely variable amounts even in mice fully protected by live virus. None of the mouse sera produced precipitates in gel diffusion tests with the soluble antigen of vaccinia virus.

Interferon in mice

Although VN antibody was present in the blood of mice receiving protective doses of live virus none was detected in 10% brain homogenates of the same mice either before or 24 hr. after intracerebral (i.c.) challenge. However, interferon-like substances were found in the brain homogenates after challenge, at a dilution of 1/18 in normal mice and 1/120 in vaccinated mice. In both cases the titres in pre-challenge samples were $< 1/5$. The results suggest that interferon is probably involved in protection.

Immunogenicity in rabbits

The challenge virus doses causing confluent or semiconfluent lesions on the skin of control animals produced only scanty or single lesions in animals immunized with most of the inactivated vaccines, indicating that they conferred a considerable degree of skin immunity, both 1 and 3 weeks after immunization (Table 2). Except for one rabbit receiving heat-inactivated vaccine, the responses to all inactivated vaccines were similar.

Lesions in immunized animals developed like those in the unimmunized up to the 5th day, but thereafter their evolution was accelerated. The individual pocks remained circumscribed, with moderate erythema surrounding them. There was no secondary spread, their mildly necrotic centres developed eschars by the 7th day, and healing preceded that of control animals by several days. In no case was there any evidence of the severe necrosis described in similar experiments by McNeill (1966). No lesions were produced in rabbits immunized with live virus.

Induction of dermal sensitivity

In rabbits injected with inactivated vaccines and tested intradermally with vaccine killed by the same method, the reactions were either trivial or absent. β-Propiolactone-inactivated vaccine induced early reactions in homologously immunized and unimmunized rabbits, but none of these developed into typical vaccinial lesions. Apart from this reaction, there were none with the other killed vaccines that could be associated with the use of a particular inactivator.

However, marked erythema and induration were produced by all the killed vaccines on the skins of rabbits previously immunized with live virus. They appeared after 24 hr. and persisted for almost 5 days before fading. Similar reactions are reported in man with killed vaccine administered to already vaccinated

individuals (Ehrengut, 1968). The reactions must be due to viral components in the test vaccine, since any host protein in the immunizing vaccine was different from that in the challenge vaccine.

Table 2. *Dermal response of individual rabbits immunized with live and inactivated vaccines*

Inactivation method for immunizing vaccine	Dose of live CAM virus (pk.f.u. by 10^{-3})			
	30	6	1·2	0·24
Heat	C	Sc	2	0
	3	2	0	0
	8	1	0	0
	2	0	0	0
β-Propiolactone	3	1	0	0
	2	0	0	0
	1	0	0	0
	1	0	0	0
Formalin	3	1	0	0
	1	0	1	0
	2	1	0	0
		Fur overgrown		
Photodynamic	3	1	0	0
	3	1	0	0
	6	2	0	0
	2	0	0	0
Ultraviolet	5	0	1	0
	1	0	0	0
	1	0	0	0
	2	0	0	0
Hydroxylamine	3	1	0	0
	2	0	0	0
	1	0	0	0
	1	0	0	0
None (live virus)	0	0	0	0
	0	0	0	0
		NT		
		NT		
No vaccine	C	Sc	1	0
	C	Sc	Sc	4
	C	C	3	1
	C	Sc	7	0

C, Sc = Confluent or semi-confluent lesions.
Numerals = Numbers of discrete pocks.
NT = Not tested.
The first two rabbits in each group challenged 1 week, the second two rabbits 3 weeks, after immunization.

Antibody responses

Antibodies were absent from all preimmunization sera. The virus-neutralizing antibody response to the killed vaccines was substantial, except after heat-treated

virus. The CF antibody response was moderate. HI antibody was usually absent (Table 3). HI antibody is considered to be a response to viral replication (Kaplan, 1962); with some anomalies, the present results confirm this, though large doses of inactivated vaccine can induce HI responses (Madeley, 1968).

There is little correlation between serum antibody concentration measured by the three methods; they are unrelated to the skin immunity induced in the same rabbits (Table 2), nor do they indicate the superiority of a particular inactivator, except perhaps BPL, in preserving the antigens that elicit them. The antibody

Table 3. *Antibody responses of individual rabbits to live and inactivated vaccines**

Inactivation method for immunizing vaccine	After immunization			After challenge†		
	VN‡	HI‡	CF‡	VN	HI	CF
Heat	0·7	< 10	40	1882	160	300
	11·0	< 10	40	8710	80	300
	1·3	< 10	< 10	9·6	< 10	30
	3·0	< 10	80	5·6	10	60
β-Propiolactone	50	< 10	100	3040	80	320
	1064	40	120	5548	320	180
	513	20	—	515	10	120
	355	< 10	80	427	20	80
Formalin	76	< 10	100	9772	160	180
	68	< 10	50	724	40	160
	32	< 10	40	80	< 10	60
	25	< 10	40	45	< 10	60
Photodynamic	21	< 10	40	1000	80	180
	69	< 10	60	760	10	120
	13	< 10	40	14	< 10	60
	3·2	< 10	30	4·6	< 10	40
Ultraviolet	69	40	40	5495	320	300
	87	10	30	1445	40	120
	29	20	80	275	20	140
	550	< 10	80	392	10	60
Hydroxylamine	115	< 10	< 10	1178	20	120
	38	< 10	30	119	< 10	60
	72	< 10	30	105	< 10	60
	19	< 10	30	101	< 10	50
None (live virus)	263	< 10	120	2970	160	240
	263	40	—	747	40	240
	1148	160	240	1995	40	240
	631	160	180	2098	20	240
No vaccine	< 0·05	< 10	< 10	903	160	240
	< 0·05	< 10	< 10	3706	160	320
	< 0·05	< 10	< 10	5·9	< 10	< 10
	< 0·05	< 10	< 10	0·6	< 10	< 10
Standard antibody	1000	80	300	1000	40	300

* The responses refer to the same rabbits as in Table 2.

† The first two rabbits in each group were challenged by dermal scarification with live virus, the second two by intradermal injection of homologous killed vaccine.

‡ See Table 1.

response obtained with BPL-inactivated virus was comparable with that of live virus. However consistent inactivation was difficult to achieve with BPL and, as described for mice, more vigorous treatment destroyed antigenicity.

Gel precipitation

Pooled sera from the groups of rabbits immunized with the inactivated vaccines gave no precipitates with soluble antigen in agar gel diffusion tests. A single broad line occurred with serum pools diluted 1/2 from rabbits immunized with live virus, and with 1/8 hyperimmune sheep serum. The production of visible precipitates thus requires serum of high antibody content. Such sera were obviously not produced by inactivated vaccines using the immunization schedule described here. The effect of any particular inactivation method on precipitating antigen cannot therefore be inferred from these results.

DISCUSSION

The kinetics of inactivation of viruses by heat, formalin, ultra-violet irradiation and dye-sensitized photoinactivation is already well documented (Woese, 1960; Gard, 1960; Taylor, 1960; Wallis & Melnick, 1965). β-Propiolactone reacts with all protein radicals and is commonly used to kill viruses for vaccines (Lo Grippo, 1960). It has seldom been used with vaccinia virus (Dostal, 1962). In our hands, inactivation was not easily controlled and requires further investigation.

Experimental vaccines inactivated by hydroxylamine have been prepared from fowl pest, influenza and foot and mouth disease viruses (Schäfer & Rott, 1962; Fellowes, 1966). Viruses differ considerably in their susceptibility to this reagent (Franklin & Wecker, 1959), and that of vaccinia virus has rarely been tested (Friedberger & Yamamoto, 1909; McNeill, 1965). We were unable to inactivate our virus suspensions completely at the concentration and exposure times recorded by McNeill. Hydroxylamine activity varies with electrolyte content and, in our hands, required approximately 0·1 M of the neutral salt for complete inactivation (Freese, Bautz-Freese & Bautz, 1961).

Heat, formalin and β-propiolactone can be considered as predominantly affecting the protein envelope of vaccinia virus. Ultraviolet inactivation, dye-sensitized photoinactivation and hydroxylamine inactivation of many viruses results principally from damage to the viral nucleic acid or nucleoprotein rather than the envelope protein, although it is doubtful whether this is always so (Turner & Kaplan, 1965; Kimes & Bussell, 1968). Absolute specificity of the site of action of the reagents is unlikely and the complex interrelated architecture of the pox viruses makes it doubtful that one site could be attacked without other modification of the whole structure (Gard, 1960). Pox virus inactivated by many of the commonly used methods can be reactivated after apparent loss of viability (Fenner, 1962; Abel, 1963; Kim & Sharp, 1967) and strains vary in resistance to some of the reagents (Sitnikov & Ghendon, 1968). Complete inactivation implies some irreversible change in nucleic acid since viral multiplication ultimately depends upon the integrity of this component. The antibody responses do not

illuminate these modes of action, apart from that of heat treatment, nor do they show the superiority of any particular inactivant.

Attempts to devise a mouse protection test for inactivated smallpox vaccine with a respiratory challenge were unsatisfactory (G. S. Turner, unpublished observations). The intracerebral challenge route used in the present experiments is fairly remote from natural infection and immunity to it is induced only by very large immunizing doses of killed vaccines (Bronson & Parker, 1944). The host factors that determine the resistance of mice against vaccinial infection are complex. Our observations offer no means of relating humoral antibody response to protection against intracerebral challenge. The suggestion that mechanisms other than orthodox specific immunity reactions are involved (Andrewes, Elford & Niven, 1948) are supported by observations of the role of interferon in vaccinia infection of mice (Finter, 1966). Our experiments suggest that intracerebral challenge may recall previously stimulated interferon (Baron, Buckler, Friedman & McCloskey, 1966); and since interferon is commonly elicited with inactivated virus it is likely that at least some of the protection afforded by the killed virus vaccines is due to this mechanism. Cellular immunity to vaccinia infection is demonstrable in mice (Hochstein-Mintzel, 1969) and is independent of antibody or interferon production. Its effects on intracerebrally injected virus are doubtful (Hirsch, Nahmias, Murphy & Kramer, 1968). We did not examine cell-mediated immune responses to our killed vaccines, but their importance in protection against pox virus infection is stressed by Boulter (1969).

We found no hypersensitivity to the killed vaccines in rabbits. The response to live virus after killed vaccine was accelerated, as in man on revaccination, but the severe necrotizing lesions described by Ramano Rao (1962) and McNeill (1966) were not observed. This may have been due to our use of bacteriologically sterile challenge virus or to the time interval between immunization and live vaccination. No such severe reactions have been reported when live vaccine follows killed smallpox vaccine in man (Beunders *et al.* 1960; Herrlich, 1959; Kaplan *et al.* 1965; Ehrengut, 1969). The element of hypersensitivity in the responses to both primary and secondary vaccination is well known (Allison, 1967) and indeed the response to revaccination is used as an index of immunity in man. Sensitivity and immunity to vaccinia virus however are not necessarily related apart from their common antigenic origin (Craigie & Wishart, 1933). Our results differ from those of Boulter (1969) in that we have always obtained strong evidence of dermal immunity in rabbits immunized with killed vaccine when challenged with homologous virus although it was unrelated to the magnitude of the humoral antibody responses. There is a similar absence of a relationship in man (von Epp, 1961; Beunders *et al.* 1960; Kaplan *et al.* 1965; Mannweiler & Geister, 1967), although the established value of hyperimmune globulin in the protection of smallpox contacts cannot be ignored (Kempe *et al.* 1961). It has been suggested that cellular mechanisms are more important in pox virus immunity than circulating antibodies (Boulter, 1969) and it is possible that the estimation of antibody is of little value in determining the immunogenicity of killed smallpox vaccine. Boulter also showed that antibody to killed vaccine differed qualitatively from that evoked by live virus. Nevertheless

rabbits immunized with killed vaccinia virus, while not protected from infection, survived challenge with lethal doses of rabbit pox (Boulter, Zwartouw & Titmuss, 1964; Madeley, 1968). We were also able to protect mice from similar challenge with adequate doses of killed vaccines.

Formalin-inactivated vaccine is used routinely in Germany for primary vaccination at 3 years or more. At the end of 1968 there had been some 250,000 recipients; dermal reaction and general symptoms on subsequent vaccination were little modified (A. Herrlich, personal communication) and though the incidence of post-vaccinial encephalitis was little affected its mortality was substantially decreased (Ehrengut, 1969; Rohde, 1968). The efficacy of inactivated vaccines in preventing subsequent vaccination accidents in man has yet to be adequately assessed in this country.

REFERENCES

ABEL, P. (1963). Reactivation of heated vaccinia virus *in vitro*. *Zeitschrift für Vererbungslehre* **94**, 249.

ALLISON, A. C. (1967). Cell-mediated immune responses to virus infections and virus induced tumours. *British Medical Bulletin* **23**, 60.

AMIES, C. (1961). Loss of immunogenic properties of vaccinia virus inactivated by formaldehyde. *Canadian Journal of Microbiology* **7**, 141.

ANDREWES, C. H., ELFORD, W. J. & NIVEN, J. S. F. (1948). Vaccinia and ectromelia in the mouse. *British Journal of Experimental Pathology* **29**, 329.

BARON, S., BUCKLER, C. E., FRIEDMAN, R. M. & MCCLOSKEY, R. V. (1966). Role of interferon during viremia. II. Protective action of circulating interferon. *Journal of Immunology* **96**, 17.

BEUNDERS, B. W. J., DRIESSEN, J. H. & VAN DEN HOEK, C. (1960). Clinical picture and serological response to vaccination with formalinized vaccinia virus followed by scarification with active vaccine in military personnel. *Archiv für die gesamte Virusforschung* **10**, 382.

BOULTER, E. A. (1969). Protection against poxviruses. *Proceedings of the Royal Society of Medicine* **62**, 295.

BOULTER, E. A., ZWARTOUW, H. T. & TITMUSS, D. H. (1964). The immunization of rabbits with inactivated vaccinia virus. *Journal of General Microbiology* **37**, iv.

BRIODY, B. A. (1959). Response of mice to ectromelia and vaccinia viruses. *Bacteriological Reviews* **23**, 61.

BRONSON, L. H. & PARKER, R. F. (1944). Vaccinial infection in the mouse. *Journal of Immunology* **49**, 193.

COLLIER, L. H., MCCLEAN, D. & VALLET, L. (1955). The antigenicity of ultraviolet irradiated vaccinia virus. *Journal of Hygiene* **53**, 513.

CRAIGIE, J. & WISHART, F. O. (1933). Skin sensitivity to the elementary bodies of vaccinia. *Candian Journal of Public Health* **24**, 72.

DONALLY, H. H. & WEIL, A. J. (1940). Formalised vaccinia virus. Results of its use with children who had never been vaccinated previously. *Journal of Pediatrics* **17**, 639.

DOSTAL, V. (1962). Problems related to the inactivation of vaccinia virus. In *Symposium International sur la Vaccination Antivariolique*, Lyon 7–8 Dec. 1962. Lyon: Merieux.

EHRENGUT, W. (1968). Impfreaktionen und Pockenimpfschutz. *Deutsche medizinische Wochenschrift* **93**, 948.

EHRENGUT, W. (1969). Non-infectious smallpox vaccines. In: *Symposium on Smallpox*. Zagreb 2–3 September 1969.

EPP, C. VON (1961). Uber das Antikörperbild des Menschen bei normalem und gestortem Verlauf einer Pockenschutzimpfung. *Archiv für Hygiene und Bakteriologie* **145**, 256.

FELLOWES, O. N. (1966). Hydroxylamine as an inactivating agent for foot-and-mouth disease virus. *Journal of Immunology* **96**, 772.

FENNER, F. (1962). The reactivation of animal viruses. *British Medical Journal* ii, 135.

FINTER, N. B. (1966). Interferons. In *Frontiers of Biology 2*. Amsterdam: North Holland Pub. Co.

FRANKLIN, R. M. & WECKER, E. (1959). Inactivation of some animal viruses by hydroxylamine and the structure of ribonucleic acid. *Nature, London* **184**, 383.

FREESE, E., BAUTZ-FREESE, E. & BAUTZ, E. (1961). Hydroxylamine as a mutagenic and inactivating agent. *Journal of Molecular Biology* **3**, 133.

FRIEDBERGER, E. & YAMAMOTO, J. (1909). Zur Desinfektion und experimentellen Therapie bei Vaccine. *Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten* Abt. 1, Ref. **44** (suppl.), 81.

GARD, S. (1957). In *The Nature of Viruses*, p. 121. Ed. G. E. W. Wolstenholme and E. C. P. Millar, London: J. & A. Churchill Ltd.

GARD, S. (1960). Theoretical considerations in the inactivation of viruses by chemical means. *Annals of the New York Academy of Sciences* **83**, 638.

GIFFORD, G. E. (1963). Studies on the specificity of Interferon. Estimation of mouse interferon. *Journal of General Microbiology* **33**, 437.

HERRLICH, A. (1959). Über Vakzine Antigen, Versuche einer Prophylaxe neuraler Impfschaden. *Münchener medizinishe Wochenschrift* **101**, 12.

HIRSCH, M. S., NAHMIAS, A. J., MURPHY, F. A. & KRAMER, J. H. (1968). Cellular immunity in vaccinia infection of mice. Antithymocyte serum effects on primary and secondary responsiveness. *Journal of Experimental Medicine* **128**, 121.

HOCHSTEIN-MINTZEL, V. (1969). Passive und adoptive Immunisierung der Säuglingsmaus gegen Poxvirus vaccinae und variolae. Ein Beitrag zur Wertigkeitsprüfung von Pockenimpstoffen. *Zeitschrift für Immunitätsforschung und experimentelle Therapie* **138**, 71.

JANSON, C. (1891). Versuche zur Erlangung künstlicher Immunität bei Variola vaccina. *Zentralblatt für Bakteriologie und Parasitenkunde* **10**, 40.

KAPLAN, C. (1962). A non-infectious smallpox vaccine. *Lancet* ii, 1027.

KAPLAN, C., BENSON, P. F. & BUTLER, N. R. (1965). Immunogenicity of ultraviolet-irradiated non-infectious vaccinia virus-vaccine in infants and young children. *Lancet* i, 573.

KAPLAN, C., MCCLEAN, D. & VALLET, L. (1962). A note on the immunogenicity of ultraviolet irradiated vaccinia-virus in man. *Journal of Hygiene* **60**, 79.

KEMPE, C. H., BOWLES, C., MEIKLEJOHN, G., BERGE, T. O., ST VINCENT, L., SUNDARA BABU, B. V., GOVINDARAJAN, S., RATNAKANNAN, N. R., DOWNIE, A. W. & MURTHY, V. R. (1961). The use of vaccinia hyperimmune gamma-globulin in the prophylaxis of smallpox. *Bulletin of the World Health Organization* **25**, 41.

KIM, K. S. & SHARP, D. G. (1967). Influence of the physical state of formalinised vaccinia virus particles on surviving plaque titre: Evidence for multiplicity reactivation. *Journal of Immunology* **99**, 1221.

KIMES, R. C. & BUSSELL, R. H. (1968). Nucleic acid type and effect of pH and hydroxylamine on canine distemper virus. *Archiv für die gesamte Virusforschung* **24**, 387.

LINDENMAN, J. VON & BUSER, F. (1962). Das inaktivierte Vakzine-Antigen nach Herrlich: Versuch einer serologischen Antigenitätsprüfung am Menschen. *Pathologia et Microbiologia* **25**, 478.

LO GRIPPO, G. A. (1960). Investigations of the use of β-propiolactone in virus inactivation. *Annals of the New York Academy of Sciences* **83**, 578.

MCCARTHY, K. & HELBERT, D. (1960). A comparison of the haemagglutinins of variola, alastrim, vaccinia, cowpox and ectromelia viruses. *Journal of Pathology and Bacteriology*, **79**, 416.

MCNEILL, T. A. (1965). The antibody response of rabbits to inactivated vaccinia virus. *Journal of Hygiene* **63**, 525.

MCNEILL, T. A. (1966). The development of skin resistance and hypersensitivity following inactivated virus vaccines in rabbits. *Journal of Hygiene* **64**, 23.

MADELEY, C. R. (1968). The immunogenicity of heat-inactivated vaccinia virus in rabbits. *Journal of Hygiene* **66**, 89.

MAHNEL, H. (1960). Tierexperimentelle Untersuchungen mit Vakzine-Antigen. *Archiv für die gesamte Virusforschung* **10**, 529.

MANNWEILER, E. & GEISTER, R. (1967). Das Verhalten der Serumantikörper bei Wiederimpfung mit Vakzinevirus. *Deutsche medizinische Wochenschrift* **92**, 2168.

PARKER, R. F. & RIVERS, T. M. (1936). Immunological and chemical investigations of vaccine virus. III. Response of rabbits to inactive elementary bodies of vaccinia and to virus-free extracts of vaccinia virus. *Journal of Experimental Medicine* **63**, 69.

RAMANO RAO, A. V. (1962). The immunogenicity of inactivated vaccinia virus. *Journal of Pathology and Bacteriology* **84**, 367.

ROHDE, W. (1968). Versuch einer Gegenuberstellung bisheriger Unterlagen einer Einschätzung der Enzephalitis-prophylaxe mittels Vakzineantigen in der D.D.R. *Zeitschrift für ärtzliche Fortbildung* **62**, 177.

RONDLE, C. J. M. & DUMBELL, K. R. (1962). Antigens of cowpox virus. *Journal of Hygiene* **60**, 41.

ROSENAU, M. J. & ANDERVONT, H. B. (1931). Vaccinia: susceptibility of mice and immunologic studies. *American Journal of Hygiene* **13**, 728.

SCHÄFER, W. & ROTT, R. (1962). Herstellung von Virus Vaccinen mit Hydroxylamine; verlauf das Inaktivierung und Wirkung des Hydroxylamins auf verschiedene biologische Eigenschaften einiger Viren. *Zeitschrift für Hygiene und Infektionskrankheiten* **148**, 256.

SELIGMANN, E. B. JR (1966). *World Health Organization Monograph Series* No. 23, p. 146.

SITNIKOV, B. S. & GHENDON, YU Z. (1968). Resistance of variola-vaccinia subgroup of viruses to treatments predominantly damaging the protein envelope or the nucleic component of the virus. *Voprosy Virusologii* **13**, 554.

SUBRAHMANYAN, T. P. & MIMS, C. A. (1966). Fate of intravenously administered interferon and the distribution of interferon during virus infections of mice. *British Journal of Experimental Pathology* **47**, 168.

TAYLOR, A. R. (1960). Effects of non-ionizing radiations on animal viruses. *Annals of the New York Academy of Sciences* **83**, 670.

TURNER, G. S. (1967). Respiratory infection of mice with vaccinia virus. *Journal of General Virology* **1**, 399.

TURNER, G. S. & KAPLAN, C. (1965). Observations on photodynamic inactivation of vaccinia virus and its effect on immunogenicity. *Journal of Hygiene* **63**, 395.

WALLIS, C. & MELNICK, J. L. (1965). Photodynamic inactivation of animal viruses: A Review. *Photochemistry & Photobiology* **4**, 159.

WEIL, A. J. & GALL, L. S. (1940). Studies on the immunization of rabbits with formalinized vaccine virus. *Journal of Immunology* **38**, 1.

WOESE, C. (1960). Thermal inactivation of animal viruses. *Annals of the New York Academy of Sciences* **83**, 741.

J. Hyg., Camb. (1970), **68**, 211
Printed in Great Britain

Direct complement fixation test with avian infectious bronchitis virus in chickens

By P. K. UPPAL

Department of Animal Pathology, University of Cambridge, Cambridge

(*Received* 19 *November* 1969)

SUMMARY

The direct complement fixation test was performed to follow the antibody response in chickens infected with avian infectious bronchitis virus. Concentrated allantoic fluid (4 units) was used as an antigen and allowed to react with serially diluted antiserum in the presence of two complete units of guinea-pig complement for 3 hr. at 4° C. and $\frac{1}{2}$ hr. at 37° C. before the addition of sensitized cells. Serum was unheated and used either fresh or within one month of storage at −30° C. Individual birds showed a rise and fall of complement-fixing antibody both after primary and secondary inoculations. The complement-fixing antibody was detected as early as the seventh day after primary inoculation. The highest complement fixation titre (1/32 to 1/64) was recorded from 14 to 21 days after inoculation with a subsequent gradual decline.

The results of the direct complement fixation tests have been correlated with the serum neutralization test. The neutralizing antibodies usually appeared by the 14th day but were not detected at a significant titre until the 21st day after primary inoculation. Serum neutralizing antibodies were still present at high titres even after 7 weeks of infection but the complement-fixing antibodies had disappeared by that time.

INTRODUCTION

Chicken or turkey sera failed to fix guinea-pig complement in the presence of homologous antigen, when heated at 56° C. for 30 min. (Bushnell & Hudson, 1927; Rice, 1947; Rice, Magwood & Annau, 1960; Nitzschke, 1954, 1956; Brumfield & Pomeroy, 1957). This finding led Rice (1948) to use an indirect complement fixation test for demonstrating antibody in heated avian antisera. However, Nitzschke (1954, 1956) developed a direct complement fixation test using sera of hens immunized with three different viral agents, viz. Newcastle disease, fowl plague and swine influenza viruses. Results obtained with direct complement fixation and inhibition complement fixation were substantially the same but the direct complement fixation test was less sensitive in distinguishing the viruses. A very few unheated chicken serum samples in the presence of guinea-pig complement (1·8 units) showed very slight anticomplementary activity at low dilutions. Brumfield & Pomeroy (1957) employed sufficient amounts of guinea-pig complement to overcome the anticomplementary effects of unheated turkey or chicken sera. They

studied ornithosis, infectious bronchitis and Newcastle disease viruses of birds and showed that the fixation was specific due to antibody rather than to the combined anticomplementary effects. Brumfield, Benjamin & Pomeroy (1959) subsequently developed a modified form of direct complement fixation test for the study of ornithosis in turkeys, which was based on the addition of unheated normal chicken serum to the test. By this technique they showed that the titres of these sera could increase two- to eightfold as compared to the titres obtained in conventional direct complement fixation tests. Rice *et al.* (1960) adopted this modified complement fixation test in favour of the indirect test when detecting antibodies to salmonella antigens in turkey sera. Contrary to the above findings, Orlans, Rose & Clapp (1962*b*) could not demonstrate the fixation of guinea-pig complement by fowl antibody against bovine serum albumin, either with fresh antiserum or with heated antiserum in the presence of normal chicken serum. This paper describes an experiment in which the antibody responses of individual birds during the course of infection with avian infectious bronchitis virus were measured both by direct complement fixation and by serum neutralization tests. The findings provide further evidence for the specificity of the direct complement fixation test for infectious bronchitis.

MATERIALS AND METHODS

Virus

The strain of avian infectious bronchitis virus isolated by Dr H. P. Chu and designated Cambridge 163/57 was used in the present study.

Experimental infection in chickens

Chickens were obtained from the flock kept at the isolation farm at the School of Veterinary Medicine, and were known to be free from avian infectious bronchitis virus. Thirty-four 8-week-old chickens were divided into two equal groups and were kept in an isolation block. All birds were bled before infection. The first group was inoculated with 0·25 ml. of undiluted infectious bronchitis virus (titre of the virus $10^{6·5}$ EID 50/ml.) in the sinuses and trachea. The second group received the same concentration of virus intravenously. Two chickens from each group were killed on the 2nd, 5th, 7th, 10th, 14th and 21st day after infection. In addition, four chickens were also bled on the 5th, 7th, 10th, 14th, 28th, 35th, 42nd and 49th day. Both complement fixation and serum neutralization tests were performed on all the serum samples. In order to study the secondary response a second dose of virus consisting of 0·4 ml. of the same concentration was given intravenously to the remaining birds of each group on the 49th day. Chickens were again bled on the 7th, 14th and 21st day following reinfection. A complement fixation test was performed on all the sera, and a serum neutralization test on two chickens from each group. The chickens were bled from the wing vein, using sterile all-glass syringes, before and after infection. The serum was usually taken from the clot on the same day as the birds were bled, and stored unheated at $-30°$ C.

Complement fixation tests

Sheep erythrocytes

Throughout the study one sheep whose cells were not fragile was used for both the preparation of haemolysin and indicator system. One volume of blood collected aseptically was added to 1·2 volumes of modified Alsever's solution (Bukantz, Rein & Kent, 1946).

Haemolysin

Two young rabbits were inoculated subcutaneously with 0·5 ml. of a 10% suspension of sheep red blood corpuscles and were observed for 10 min. for any untoward reaction. Neither of the rabbits showed any reaction, and 1 ml. of a 10% suspension of sheep red blood cells was then inoculated intravenously into each. This was followed by 1, 2, 2·5, 3 and 5 ml. amounts of a 20% suspension given intravenously at intervals of two days. Rabbits were bled for testing a week after the last injection and the antibody titres were found to be satisfactory. Serum was heated at 56° C. for 30 min. and preserved in equal volumes of sterile neutral glycerine, bottled in small ampoules and stored at 4° C. Minimum haemolytic dose (MHD) of haemolysin was determined. The highest dilution of haemolysin showing complete haemolysis was taken as one unit. The titre of the haemolysin was 1/16,000.

Sensitized cells

After washing three times, the sheep red cells were packed at 2000 rev./min. for 10 min. A 4% suspension of the cells was made in veronal buffer solution (pH 7·4) (Mayer, Osler, Beir & Heidelberger, 1946) and an equal volume of this suspension was mixed with an equal volume of dilute haemolysin containing 4 MHD. The mixture was shaken thoroughly and kept in a water bath at 37° C. for 20 min. The sensitized cells were always prepared fresh before use.

Complement

Young male guinea-pigs were first bled individually and their sera tested for lytic activity against sheep erythrocytes. Only those guinea-pigs whose sera did not have any lytic activity were used. Clear serum collected from six to eight guinea-pigs was pooled and preserved by the addition of an equal volume of a solution containing 12% sodium acetate and 4% boric acid in distilled water (Sonnenschein, 1930). The preservative was tested for traces of zinc with 0·1% sodium-diethyl-dithio-carbamate. The complement was titrated in the presence of antigen and normal fowl serum under the conditions of the tests. The highest dilution of complement showing complete lysis was taken as one unit of complement.

Antigen

Fertile eggs 9–10 days old were inoculated in the allantoic sac with 0·1 ml. of a 10^{-2} dilution of virus (titre of the virus $10^{6.5}$ EID 50/ml.) and were incubated at 37° C. Eggs dying within 24 hr. were discarded. The eggs were chilled after

48 hr. of virus multiplication, the allantoic fluids harvested aseptically, pooled and centrifuged at 3000 rev./min. for 15 min. to remove the gross particles. The clear allantoic fluid was further centrifuged at 19,000 rev./min. for 1 hr. The resulting pellets were resuspended in a small quantity of veronal buffer and subjected to vibration at 1·5 amperes (MSE ultrasonic power unit) for 1 min. The suspension was then distributed in about 1·5 ml. volumes and kept frozen at −30° C. In practice the pellet yield from 1500 ml. of allantoic fluid obtained from 150 eggs was resuspended in 15 ml. of veronal buffer. The optimal concentraction of antigen was determined in the presence of positive serum by the chess board titration method. It was observed that the antigen in a dilution of 1/8 was quite suitable for the actual complement fixation test.

Sera

Sixteen normal chickens were bled in order to study the anticomplementary activity of their sera. The tests were done on fresh unheated sera and also after storage at 4° C. and −30° C. Twofold dilutions of each fresh unheated serum were made ranging from 1/8 to 1/128. Diluted sera in 0·2 ml. volumes were distributed in seven different rows of tubes and 2 units of complement in 0·2 ml. were added to each followed by 0·2 ml. of diluent in place of antigen. The system was incubated at 4° C. One row of the tubes was taken from the refrigerator at 1, 2, 3, 4, 6, 12 and 18 hr. and incubated at 37° C. for ½ hr. The system was further incubated for ½ hr. at 37° C. after the addition of sensitized cells. The results indicated that eight sera after 6 hr. and 11 after 12 hr. were anticomplementary. Similarly serum samples after 1 month of storage at 4° C. and −30° C. were tested as described above except that the system was incubated at 4° C. for 3 hr., a period at which none of the fresh unheated sera were anticomplementary. It was shown that after storage for 1 month at 4° C. seven of the 16 sera were anticomplementary, but after storage at −30° C. none of the sera were anticomplementary.

Test proper

Serial twofold dilutions of unheated chicken sera in 0·2 ml. volumes were made in round-bottomed tubes starting from a 1/8 dilution, 0·2 ml. of antigen (4 units) was added followed by 0·2 ml. of complement (2 units). The suitable controls of antigen, antiserum and complement were included. The system was kept in the refrigerator at 4° C. for 3 hr. with occasional shaking followed by further incubation for ½ hr. in a 37° C. water bath. Sensitized cells in volumes of 0·2 ml. were added to each tube and the results were read after 30 min., when the antigen, antisera and complement controls showed complete haemolysis. Positive and negative sera were used to ensure that the sensitivity of the successive tests were uniform. The highest dilution of the serum showing more than 50 % fixation was taken as the end titre of the serum. The results were read visually.

Serum neutralization test

Initial dilutions of 1/2 and 1/5 of heated sera (56° C. for 30 min.) were made. Serial twofold dilutions of 1/5 sera were subsequently made to 1/320. Constant

amounts of virus (0·05 ml. containing 100 EID 50) were mixed with equal volumes of the diluted sera. The mixture was kept at 4° C. for 1 hr. and then inoculated in 0·1 ml. volume into the allantoic sac of each of five 9-day-old fertile eggs which were incubated at 37° C. for 8 days. Death and dwarfing of the embryos was regarded as the indication of infection by the virus. Those eggs dying within 24 hr. after infection were discarded. The highest dilution of serum showing more than 50 % virus neutralization was taken as the titre of the serum. Positive and negative serum controls were always included in the experiments. Whenever there was a deviation in the titre of the positive control serum by more than one tube the test was repeated.

RESULTS

The antibody responses in chickens infected with infectious bronchitis virus both by respiratory and intravenous routes have been demonstrated both by direct complement fixation and serum neutralization tests as shown in Tables 1 and 2 and

Table 1. *Complement-fixing and neutralizing antibody titres in sera of chickens killed at various times after intratracheal and intranasal inoculation of avian infectious bronchitis virus*

Days after infection	Chicken no.	Antibody titre: Complement fixing	Antibody titre: Neutralizing
0	601	< 8	< 2
2	604	< 8	< 2
2	605	< 8	< 2
5	606	< 8	< 2
5	607	< 8	< 2
7	609	8	< 2
7	667	< 8	< 2
10	611	16	< 2
10	657	16	< 2
14	676	64	2
14	669	32	< 2
21	608	64	5
21	671	16	5

Figs. 1–4. It was found that none of the sera before infection showed complement-fixing or neutralizing antibody titres. However, complement-fixing antibody was clearly shown in most of the sera by the 7th day after virus inoculation, though neutralizing antibody could not be detected before the 14th day. There was a significant rise of the complement-fixing antibodies after the 7th day of experimental infection, reaching its highest titre between the 14th and 28th day, while neutralizing antibody reached its peak after the 28th day and remained more or less constant up to the 49th day, the maximum period tested. The complement-fixing antibody tends to disappear after the 28th day and was negative in most of the sera collected on the 42nd and 49th day after infection. After a second inoculation there was a rise both in complement-fixing and neutralizing antibody titres.

Table 2. *Complement-fixing and neutralizing antibody titres in sera of chickens killed at various times after intravenous inoculation of avian infectious bronchitis virus*

Day after infection	Chicken no.	Antibody titre: Complement fixing	Antibody titre: Neutralizing
0	600	< 8	< 2
2	602	< 8	< 2
2	603	< 8	< 2
5	656	< 8	< 2
5	683	< 8	< 2
7	655	8	< 2
7	659	8	< 2
10	661	16	< 2
10	663	32	< 2
14	672	32	2
14	654	32	< 2
21	658	32	5
21	669	32	5

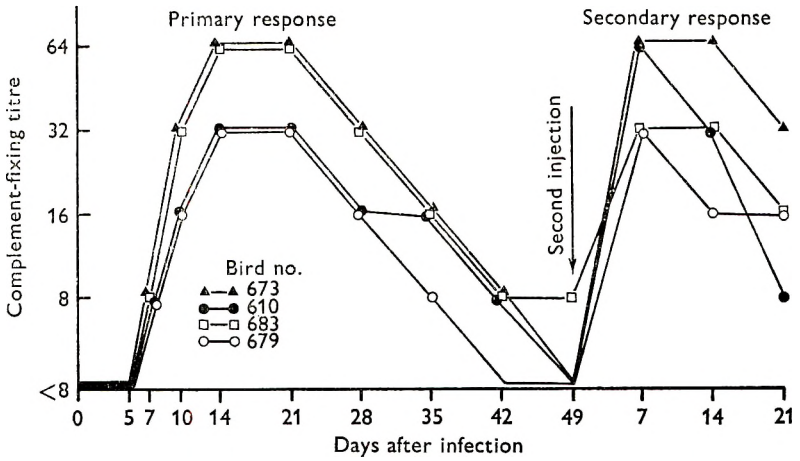

Fig. 1. Development of complement-fixing antibodies in chickens infected in the respiratory tract with avian infectious bronchitis virus.

DISCUSSION

In the present study it has been observed that the concentration of the antigen, dosage of complement, collection and storage of chicken serum and the period of interaction between antigen and antibody are all critical for the successful application of the direct complement fixation test. Allantoic fluid as an antigen without concentration failed to fix complement in the presence of homologous antibody. However, the allantoic fluid when centrifuged at high speed yielded a suitable antigen for the complement fixation test. Nitzschke (1954) also used six times concentrated allantoic fluid infected with Newcastle disease virus as an antigen for the direct complement fixation test. Brumfield & Pomeroy (1957) also

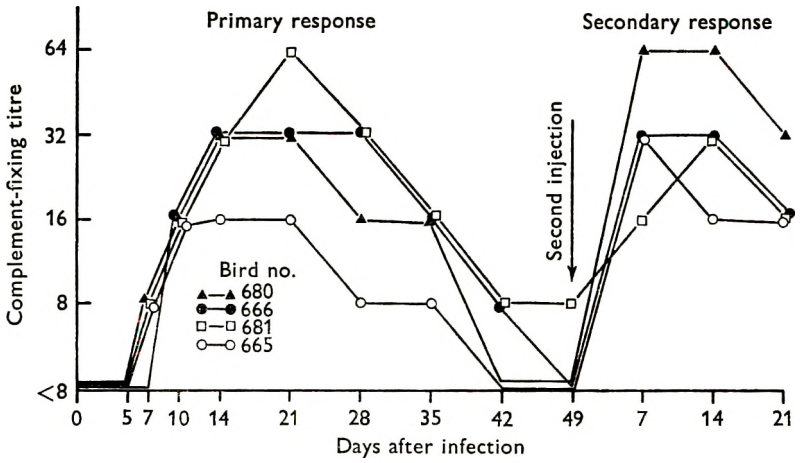

Fig. 2. Development of complement-fixing antibodies in chickens infected intravenously with avian infectious bronchitis virus.

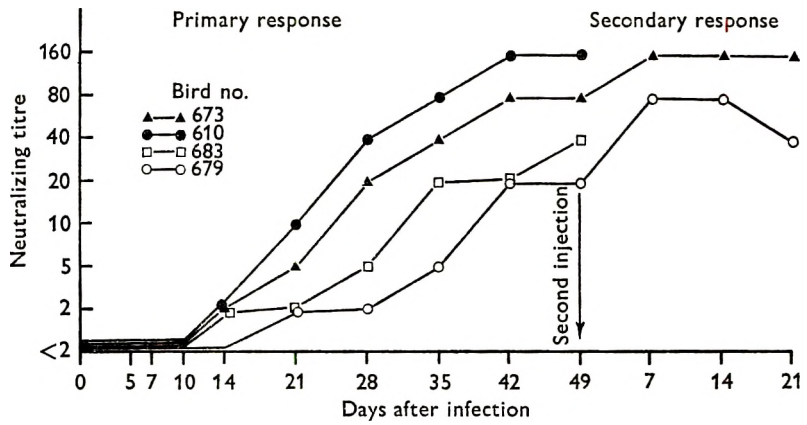

Fig. 3. Development of neutralizing antibodies in chickens infected in the respiratory tract with avian infectious bronchitis virus.

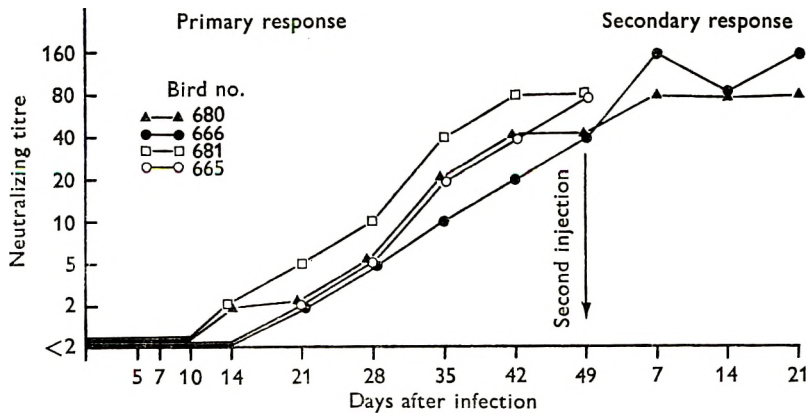

Fig. 4. Development of neutralizing antibodies in chickens infected intravenously with avian infectious bronchitis virus.

noticed that the allantoic fluid harvested from eggs infected with infectious bronchitis virus, when used without concentration, failed to give reliable results.

None of the unheated chicken sera in the presence of two complete units of guinea-pig complement showed any anticomplementary activity when tested fresh or after storage for a month at $-30°$ C. Similarly Brumfield & Pomeroy (1957), Tsubahara, Kataoko & Kato (1961), Harada & Matumato (1962) and Kato & Horiuchi (1965) did not find any anticomplementary activity with those unheated sera which were kept frozen at $-20°$ C. before use. Orlans, Rose & Clapp (1962*a*), however, found that even fresh fowl serum in a dilution of 1/100 inhibits the lysis of sensitized cells by guinea-pig complement. In the present experiments appreciable anticomplementary activity was observed with certain sera when stored at 4° C. Nitzschke (1954) also found that, with chicken sera which had been stored for a long period at 2° C., four out of ten showed clear anticomplementary activity. The optimum time for the fixation of complement in the presence of unheated chicken serum and antigen was found out because most of the unheated chicken sera after long storage at 4° C. were so anticomplementary that specific fixation could not be interpreted. The period of 3 hr. at 4° C. followed by incubation at 37° C. for $\frac{1}{2}$ hr. was found to be ideal for the test.

Brumfield & Pomeroy (1957) employed direct complement fixation with the sera of birds immunized with three viral agents, ornithosis, infectious bronchitis virus and Newcastle disease virus. Immune sera against heterologous antigens within the three systems gave negative results. They showed that the fixation was specific due to antibody rather than anticomplementary effects. Orlans *et al.* (1962*b*) could not use direct complement fixation tests with soluble antigen–antibody systems. They attributed their failure to the possibility that particulate antigen or antibody produced during the course of infection might provide better conditions for C^1 fixation than the soluble antigen. In the present investigation the specificity of the test is shown by following the antibody response in the individual birds infected with avian infectious bronchitis virus. None of the 34 sera collected before infection showed any complement-fixing or neutralizing antibodies. The rise and fall of the complement-fixing titres in the individual birds both after primary and secondary inoculation suggest that the development of complement-fixing antibody is associated with infection rather than any non-specific reaction. The results of the complement fixation tests were further correlated with serum neutralization tests. It was shown that the complement-fixing antibodies appeared earlier than serum neutralizing antibodies. It is presumed that the direct complement fixation test will provide a useful additional serological test for the study of infectious bronchitis.

The author wishes to thank Professor W. I. B. Beveridge for providing the necessary laboratory facilities and Dr H. P. Chu and Professor R. R. A. Coombs for valuable suggestions during the course of this work, which was carried out during the tenure of a Commonwealth Scholarship.

REFERENCES

BRUMFIELD, H. P., BENJAMIN, S. & POMEROY, B. S. (1959). Test based on normal serum component implementing fixation of turkey antiserum. *Proceedings of the Society for Experimental Biology and Medicine* **102**, 278.

BRUMFIELD, H. P. & POMEROY, B. S. (1957). Direct complement fixation by turkey and chicken sera in viral system. *Proceedings of the Society for Experimental Biology and Medicine* **94**, 146.

BUKANTZ, S. C., REIN, C. R. & KENT, J. F. (1946). Studies in complement fixation. 2. Preservation of sheep's blood in citrate dextrose mixtures (modified). *Journal of Laboratory and Clinical Medicine* **31**, 394.

BUSHNELL, L. D. & HUDSON, C. B. (1927). Complement fixation and agglutination tests for Salmonella pullorum infection. *Journal of Infectious Diseases* **41**, 388.

HARADA, K. & MATUMATO, M. (1962). Antigen relationship between mammalian and avian poxes as revealed by direct complement fixation reaction. *Japanese Journal of Experimental Medicine* **32**, 369.

KATO, K. & HORIUCHI, T. (1965). Isolation of sparrow pox and its serological properties. *National Institute of Animal Health Quarterly* **5**, 130.

MAYER, M. M., OSLER, A. G., BIER, O. G. & HEIDELBERGER, M. (1946). Activating effect of magnesium and other cations on the hemolytic function of complement. *Journal of Experimental Medicine* **84**, 535.

NITZSCHKE, E. (1954). Über den Antikörpernachweis mit der Komplementbindungs-und Komplementbindungs-Hemmungs-Reaktion bei geflugelpestinfizierten Huhnern. *Zentralblatt für Veterinärmedizin* **1**, 565.

NITZSCHKE, E. (1956). Untersuchungen über die Komplementbindungs-und Komplementbindungs-Hemmungs-Reaktion mit Huhner-Immunisieren gegen die Viren der atypischen und der klassischen Geflugelpest sowie der Schweine-Influenza. *Zentralblatt für Veterinärmedizin* **3**, 75.

ORLANS, E., ROSE, M. E. & CLAPP, K. H. (1962*a*). Fowl antibody. V. The interactions of fresh and heated fowl serum and of guinea-pig complement measured by the lysis of sensitized red cells. *Immunology* **5**, 649.

ORLANS, E., ROSE, M. E. & CLAPP, K. H. (1962*b*). Fowl antibody. VI. The fixation of complement by antibody to soluble antigens. The effect of fresh normal fowl serum on the precipitation reaction. *Immunology* **5**, 656.

RICE, C. E. (1947). Atypical behaviour of certain avian antisera in complement fixation tests. *Canadian Journal of Comparative Medicine* **11**, 236.

RICE, C. E. (1948). Studies in pullorum disease. XII. Technique of the indirect complement fixation test for activity with salmonella pullorum antigen. *Canadian Journal of Comparative Medicine* **12**, 130.

RICE, C. E., MAGWOOD, S. E. & ANNAU, E. (1960). A modified direct complement fixation test for the detection of antibodies for salmonella antigen in turkey sera. *Canadian Veterinary Journal* **1**, 132.

SONNENSCHEIN, C. (1930). Komplementkonservierung durch Natriumazetat und Borsaure. *Zeitschrift für Immunitätsforschung und experimentelle Therapie* **67**, 512.

TSUBAHARA, H., KATAOKA, T. & KATO, K. (1961). Complement fixation in fowl pox. III. Direct complement fixation with fowl serum. *Bulletin of the National Institute of Animal Health*. No. 41, 1.

J. Hyg., Camb. (1970), 68, 221
Printed in Great Britain

Comparison of materials used for cleaning equipment in retail food premises, and of two methods for the enumeration of bacteria on cleaned equipment and work surfaces

By R. J. GILBERT

Food Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9

(*Received* 12 *December* 1969)

SUMMARY

There is no official scheme for testing disinfectants and detergent/disinfectants for use in the retail food trade and few recommended procedures have been given for the cleaning of equipment with these agents. Therefore, field trials were carried out in a large self-service store. Comparisons were made of the various cleaning efficiencies, as determined by bacterial plate counts, of detergent and disinfectant solutions and machine cleaning oils applied with either clean cloths or disposable paper towels to items of equipment. The most satisfactory results were always obtained when anionic detergent (0·75 % w/v) and hypochlorite (200 p.p.m. available chlorine) solutions were applied in a 'two-step' procedure.

Tests were made to compare the calcium alginate swab-rinse and the agar sausage (Agaroid) techniques for the enumeration of bacteria on stainless steel, plastic, formica and wooden surfaces before and after a cleaning process. Although recovery rates were always greater by the swab-rinse technique, the agar sausage technique was considered to be a useful routine control method for surface sampling.

INTRODUCTION

In the report of the Aberdeen typhoid outbreak (Report, 1964) it was suggested that some form of testing scheme for the various bactericidal detergents intended for use in shops and food premises was desirable. Such a scheme might be similar to that already operated for disinfectants and detergent/disinfectants used in the dairy industry. It is possible to support these comments but still appreciate that government advisors and the manufacturers of disinfectants would find it difficult though not impossible to design a suitable test method.

The Hoy Can Test (Hoy & Clegg, 1953; Clegg, 1955) forms part of the procedure by which all disinfectants and detergent/disinfectants (often referred to as detergent/sterilizers) for use in the dairy industry are examined. The disadvantages of the Hoy Can Test, which arise from the rather complicated nature of the test and the cumbersome equipment used, led Lisboa (1959) to develop a method in which the 10 gal. (Hoy & Clegg, 1953) or 5 gal. (Cousins, Hoy & Clegg, 1960) milk cans were replaced by stainless steel tubes. In their present form the Hoy Can and Lisboa Tube Tests do not appear to have been employed for evaluating disin-

fectants and detergent/disinfectants for use in the retail food trade. However, attempts could be made to modify this type of test to make it more applicable, i.e. to make the conditions of the test simulate those encountered in practice in food establishments. Although such a testing scheme is desirable for the approval of disinfectants it is also necessary to have actual 'field-tests' on retail premises to determine the suitability of the material.

Part I of this paper describes a comparative assessment, based on bacteriological tests only, of some types or combinations of detergents, disinfectants and detergent/disinfectants for cleaning items of equipment in retail food premises. Most disinfectants and detergent/disinfectants used in the retail food trade contain compounds which release chlorine or quaternary ammonium compounds. There are numerous products on the market with activity based on these two compounds; representative types only were tested.

After the cleaning and disinfection of equipment and surfaces it is necessary to have a simple method of assessing the efficiency of the procedures. A visual inspection is always useful and Brookes & Fennell (1952) developed a powder-dusting technique which was easy to apply and indicated the presence of grease or film on eating utensils. They claimed that the powder-dusting technique gave better control of dish-washing and a more sensitive differentiation of the performance of detergents than would be possible by bacteriological tests. Nevertheless, the powder-dusting technique does not appear to have been widely used.

The various techniques for the microbiological examination of surfaces have been reviewed by Favero *et al.* (1968). The basic methods can be classified as follows: (i) the swab-rinse technique; (ii) rinse-tests; (iii) agar contact methods; and (iv) direct counts from surface agar plating. Other modifications of these methods have been described in the literature. In this country the swab-rinse technique using cotton wool or calcium alginate swabs and an agar contact method using 'agar sausages' (Agaroid, Oxoid Ltd, London, S.W. 1) are commonly employed. A short comparative study of the enumeration of bacteria on surfaces by these two methods is given in Part II of this paper.

PART I

Comparison of various agents used for cleaning equipment in retail food premises

All tests were made at one large modern self-service store by arrangement with the directors of the company and the manager. The store was clean and well organized and the staff had been given instruction on hygienic methods of food handling. Twenty-four visits were made over the period March–September 1968, usually at weekly intervals.

Duplicate areas (each 30 cm.^{2}) from the upper and lower surfaces of the blade of a cooked-meat slicing machine were swabbed using two calcium alginate swabs/area (Higgins, 1950): the technique used has been described previously (Gilbert & Maurer, 1968). The same technique was used for swabbing one side of a large carving knife blade (80 cm.^{2}) and a bench mounted can-opener (1 cm.^{2}

blade and about 4 cm.^{2} area above the blade). Four swabs were used for the carving knife and two for the can-opener. Inactivating agents were added to the quarter-strength Ringer's solution used as diluent, 0·5 % w/v sodium thiosulphate when the cleaning agents contained 200 parts per million (p.p.m.) of available chlorine, and 0·5 % v/v Tween 80 (a sorbitan mono-oleate) when the cleaning agent contained a quaternary ammonium compound. Sodium thiosulphate was

Table 1. *The types and concentrations of detergent, disinfectant, detergent/disinfectants and machine cleaning oils used, and times taken to complete the cleaning procedure*

Code*	Cleaning agent used		Applied with	Time for cleaning (min.)
	Type	Concentration		
A	Detergent (anionic)	0·75 % w/v	Clean cloth	9–10‡
B	Disinfectant (hypochlorite)	200 p.p.m.†	Clean cloth	9–10‡
C	Detergent (anionic)/disinfectant (chlorinated trisodium phosphate)	0·75 % w/v 20 p.p.m.†	Clean cloth	9–10‡
D	Detergent (anionic)/disinfectant (chlorinated trisodium phosphate)	0·75 % w/v 20 p.p.m.†	Paper towel	9–10‡
E	Detergent (anionic) followed by disinfectant (hypochlorite)	0·75 % w/v, 200 p.p.m.†	Paper towel	12–13‡
F	Detergent (non-ionic)/disinfectant (quaternary ammonium compound)	0·6 % v/v	Clean cloth	9–10‡
	Machine cleaning oil (*X*)	Undiluted	Paper towel	2 §
	Machine cleaning oil (*Y*)	Undiluted	Paper towel	2 §

* The code letters A–F refer to the lettered columns in Figs. 1 and 2.

† p.p.m. = parts per million of available chlorine.

‡ These times include the filling of buckets with hot water, the stripping, cleaning, rinsing and reassembly of the slicing machine, and the cleaning and rinsing of the carving knife and can-opener.

§ These times refer only to the cleaning of the upper and lower surfaces of the cutting blade of the slicing machine.

not added to the diluent when the cleaning solution used contained only 20 p.p.m. of available chlorine. All plate counts were made on blood agar using a modified Miles & Misra (1938) technique with incubation for 48 hr. at 22 and 37° C.

The cleaning techniques used in the present study were described by Gilbert & Maurer (1968). Experiments were made with 'single-step' procedures using detergent, disinfectant or detergent/disinfectant solutions alone, and with a 'two-step' procedure using detergent solution followed by disinfectant solution. The effects of several detergents and disinfectants used together or separately (Table 1), applied either with disposable paper towels or freshly cleaned and disinfected cloths, were tested on each of four separate occasions.

Some manufacturers of slicing machines recommend the use of certain vegetable

oils for the regular cleaning of their machines; such oils will also act as lubricants. An assessment of two oils (Table 1, *X* and *Y*) was made to determine whether, when applied with disposable paper towels, they were as efficient in a cleansing procedure as detergent, disinfectant or detergent/disinfectant solutions.

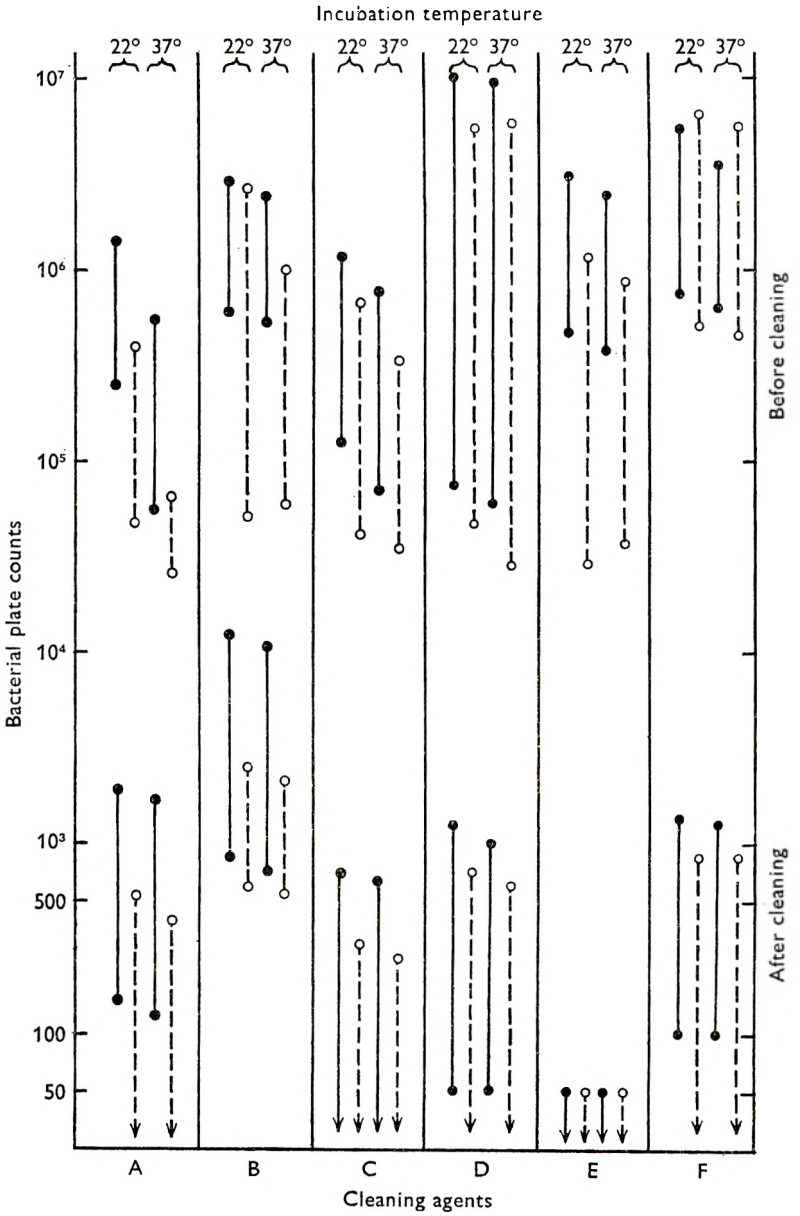

Fig. 1. Range of bacterial plate counts at 22 and 37° C. from swabbed areas of upper and lower surfaces of a slicing machine blade, before and after cleaning. ●——● = Upper surface of blade; ○- - -○ lower surface. A–F, different cleaning agents used. For explanation see Table 1.

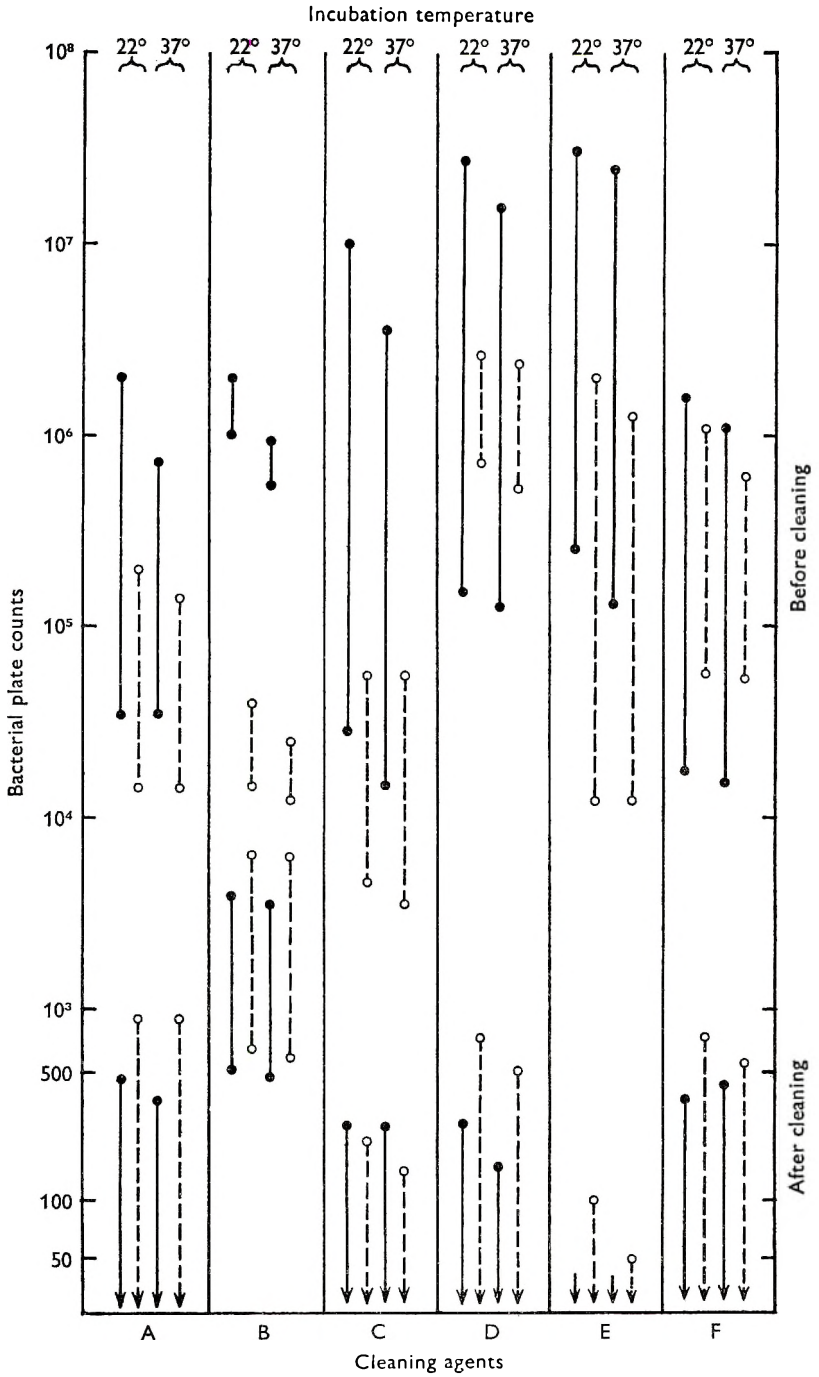

Fig. 2. Range of bacterial plate counts at 22 and 37° C. from swabbed areas of a carving knife blade and a can-opener, before and after cleaning. ●——● = Carving knife blade; ○- - -○ = can-opener. A–F, different cleaning agents used. For explanation see Table 1.

RESULTS

It is more difficult to standardize field-tests on cleaning and disinfecting agents than *in vitro* tests carried out in a laboratory. Reasonable care was taken to standardize the conditions in the self-service store and tests were replicated whenever possible. The items tested had all been cleaned at about 5 p.m. on the previous day by the staff normally responsible, and they had been in use for at least 3 hr. on the day when tests were made. The number of cooked and canned meats cut on the slicing machine before test areas were sampled was in the range of 17–25 (mean 20)/visit: the most popular products sliced on the machine were ham, pork

Table 2. *Experiments with machine cleaning oils*

(Bacterial plate counts at 22 and 37° C. from swabbed areas of slicing machine blades before and after cleaning.)

		Plate counts/swabbed area					
				After cleaning with oil			
		Before cleaning		*X*		*Y*	
Object swabbed	Expt.	22° C.	37° C.	22° C.	37° C.	22° C.	37° C.
Upper surface of blade	1	162,000	87,500	6625	4750	N.D.	N.D.
	2	250,000	56,250	17,500	11,250	N.D.	N.D.
	3	125,000	70,000	N.D.	N.D.	3750	2250
	4	225,000	210,000	N.D.	N.D.	40,000	25,000
Lower surface of blade	1	525,000	362,000	41,250	23,750	N.D.	N.D.
	2	120,000	36,250	26,250	20,000	N.D.	N.D.
	3	60,000	35,000	N.D.	N.D.	4750	3250
	4	400,000	65,000	N.D.	N.D.	5500	2500

Machine cleaning oils *X* and *Y* applied with disposable paper towels.
N.D. = not done.

luncheon meat, corned beef, jellied veal, ox tongue, savoury sausage, liver sausage, continental sausage and salami. The can-opener had been used for opening many cans of meat and the carving knife for cutting or slicing various types of savoury, liver or continental sausage and salami.

Fig. 1 shows the range of plate counts from swabbed areas of the slicing machine after normal use and after treatment with solutions of the cleaning and disinfecting agents applied with clean cloths or paper towels. Each count represents the mean from duplicate swabbed areas. Counts were usually higher after incubation at 22° C. than at 37° C. After incubation, plates were examined to ascertain the types of bacteria present. Before cleaning, micrococci, coliform and aerobic sporing bacilli were usually found and, on several occasions, α-haemolytic and non-haemolytic streptococci, *Klebsiella* spp. and *Proteus* spp. were also isolated. No effort was made to isolate salmonellas as it is unusual to find these organisms in cooked or canned meats, but on two occasions coagulase-positive staphylococci were found.

The viable counts were greatly reduced by all the cleaning procedures. The most satisfactory results, i.e. lowest counts, were obtained when detergent (0·75 % w/v) and disinfectant (hypochlorite, 200 p.p.m. available chlorine) solutions were used in a two-step procedure. The least satisfactory results, i.e. highest counts, were obtained when a solution of disinfectant (hypochlorite, 200 p.p.m. available chlorine) alone was used.

Fig. 2 shows the range of plate counts from swabbed areas of the carving knife and can-opener after normal use and after treatment with solutions of the cleaning and disinfecting agents applied with clean cloths or paper towels. The most satisfactory results were again obtained when detergent (0·75 % w/v) and disinfectant (hypochlorite, 200 p.p.m. available chlorine) solutions were used in a two-step procedure.

Table 2 shows the plate counts from swabbed areas of the slicing machine after normal use and after cleaning with the two machine oils (*X* and *Y*) applied with paper towels. The results show that plate counts after cleaning with *X* or *Y* were often unsatisfactory.

Part II

Comparison of the swab-rinse and agar sausage techniques for assessing the cleanliness of equipment and surfaces in food premises

All tests were made at another large modern self-service store by arrangement with the directors of the company and the manager. The store was clean and well organized and the staff had been given instruction on hygienic methods of food handling. Five visits were made over the period September 1968–May 1969 at *ca.* 2 monthly intervals; a Senior Public Health Inspector was present on each occasion.

Three areas of a slicing machine (upper and lower surfaces of the stainless steel blade and the blade cover), a large stainless steel carving knife, a plastic display dish, a formica working surface and a wooden chopping table were selected for the tests. They were all in the area used for the preparation of cooked meats except the wooden chopping table, which, although near this preparation area, was used exclusively for the cutting and boning of raw meat. The equipment and surfaces were cleaned with a combined anionic detergent/disinfectant (20 p.p.m. available chlorine) solution and tests were made both before and after cleaning.

Four areas (each about 8 cm.^{2}) were tested using the agar sausage technique with plate count and MacConkey Agaroid sausages. The use of slices of agar medium cut from an agar sausage to grow bacteria from surfaces was first described by Ten Cate (1963, 1965). The areas within two metal templates (each about 8 cm.^{2}, i.e. similar in area to the surface of a slice from an agar sausage) were also tested, using the calcium alginate swab-rinse technique with surface plating on blood agar (Higgins, 1950) as described previously (Gilbert & Maurer, 1968). The swab-rinse technique was carried out by the author and the agar sausage technique by the Public Health Inspector. Randomly selected areas in close proximity to each other were sampled at the same time. The blood agar plates and the plate count and MacConkey Agaroid slices were incubated for 24 hr. at 37° C.

Table 3. *Comparison of the swab-rinse and agar sausage (Agaroid) techniques*

(Bacterial counts at 37° C. from test areas of shop equipment before and after cleaning.)

		Counts/test area				
		Before cleaning*	After cleaning			
				Agar sausage technique		
Object	Visit	Swab-rinse technique† B.A.	Swab-rinse technique† B.A. (P)	P.C.A.‡ (Q)	MacC.A.‡	Column (P) / Column (Q)
Slicing machine blade. Upper surface	1	105,000	800	85	80	9·4
	2	475,000	860	> 100	> 100	< 8·6
	3	225,000	112	19	21	5·9
	4	875	< 25	2	2	< 12·5
	5	1,037,000	50	6	5	8·3
Slicing machine blade. Lower surface	1	132,000	235	16	18	14·7
	2	900,000	150	13	18	11·5
	3	200,000	187	17	19	11·0
	4	4,250	< 25	4	3	< 6·2
	5	275,000	75	12	12	6·2
Slicing machine. Blade cover	1	3,250	240	16	17	15·0
	2	8,750	75	24	33	3·1
	3	20,000	25	4	4	6·2
	4	500	25	4	3	6·2
	5	22,500	< 25	2	2	< 12·5
Carving knife	1	15,000	200	39	28	5·1
	2	102,500	200	24	23	8·3
	3	5,000,000	25	4	2	6·2
	4	362,500	< 25	4	3	< 6·2
	5	4,000	25	3	2	8·3
Plastic dish	1	2,500	50	8	5	6·2
	2	62,500	30	4	4	7·5
	3	312,500	25	3	3	8·3
	4	36,250	< 25	2	2	< 12.5
	5	17,000	25	3	4	8·3
Formica working surface	1	1,250	50	7	5	7·1
	2	250	75	6	8	12·5
	3	37,500	25	8	9	3·1
	4	1,750	< 25	2	2	< 12·5
	5	2,500	25	2	2	12·5
Wooden chopping table	1	287,500	3,750	> 100	> 100	< 37·5
	2	362,500	35,000	> 100	> 100	< 350·0
	3	3,250,000	45,000	> 100	> 100	< 450·0
	4	7,500	875	20	24	43·7
	5	125,000	10,750	> 100	> 100	< 107·5

* Counts before cleaning were all uncountable (> 100) by the agar sausage technique except that from slicing machine blade cover on MacConkey agar at visit 4 (80/test area), and from formica working surface on both media at visit 2 (18/test area).

† Mean of two counts, two swabs/count.

‡ Mean from two Agaroid slices.

B.A. = blood agar; P.C.A. = plate count agar (Agaroid); MacC.A. = MacConkey agar (Agaroid).

RESULTS

Table 3 shows bacterial counts from areas of equipment and surfaces both before and after cleaning. The number of bacteria present on the stainless steel, plastic and formica surfaces was greatly reduced by the cleaning procedure, but the results for the wooden surface were far less satisfactory, as would be expected. Dilutions could be made from the rinse solutions so that viable counts were readily calculated by the swab-rinse technique. One direct count only was obtained from an agar slice and it was difficult to count > 100 colonies/slice. As a result it was usually impossible to calculate the factors by which the swab-rinse and agar sausage counts differed before cleaning, since the number of colonies on the Agaroid slices were too numerous to count. However, such factors could be calculated from the results of many of the tests carried out after cleaning. For the stainless steel surface of the blade and cover of the slicing machine, the stainless steel carving knife, the plastic display dish and the formica working surface the factors by which counts by the swab-rinse technique exceeded those by the agar sausage technique were within the range 3·1–15·0; it was usually impossible to calculate the factors for the wooden chopping table. Furthermore, differences noted were subject to the recovery media which were different for the two techniques, viz. blood agar for the swab-rinse technique and plate count agar for the agar sausage technique.

DISCUSSION

An assessment of the activities of surface active agents for use in the catering industry has been given by Hobbs, Emberley, Pryor & Smith (1960) and a description of the use of disinfectants in the food industry by Goldenberg & Relf (1967).

The choice of detergents and detergent/disinfectants for use in the retail food trade should be governed by the following factors: (*a*) they must be efficient under the conditions of use, e.g. the removal of protein and fatty material associated with meat and other food products; (*b*) they must not affect those who use them; (*c*) they must not damage or corrode equipment or working surfaces; (*d*) they must not affect the colour or flavour of food in contact with equipment and surfaces cleaned by their use; (*e*) they must be easily rinsed away; (*f*) they must be easy and safe to dispense and handle and (*g*) they must be compatible if mixed.

At the present time there seems to be no limit to the number of detergents, disinfectants and detergent/disinfectants commercially available. Instructions on the label for the use of these products are usually both clear and concise but all too often not enough consideration is given by the user to the choice of a suitable product for a particular purpose. For example, many retail premises are known to be using hypochlorite solutions without a detergent to clean equipment and working surfaces. It is imperative that workers in retail food premises should appreciate that in order to achieve cleanliness and freedom from bacteria it is not sufficient to 'disinfect' by the use of disinfectants alone; visible grease and food material must be removed by scraping and scrubbing or thorough washing with hot water containing detergent or soap. Suggested cleaning routines for surfaces, equipment (including slicing machines) and utensils in the retail meat trade have

been published recently (Food Hygiene Code of Practice, no. 8, 1969). Any disinfection procedure, e.g. with hypochlorite, should include or follow a cleaning process, and the use of combined detergent/disinfectants has become popular in retail food premises and catering establishments. The main advantage of such products is that the time taken to complete the cleaning and disinfection process is considerably reduced; the main disadvantage is that some of the disinfectant action will be rapidly lost owing to inactivation by food material. The applications of detergent/disinfectant mixtures together with their advantages and disadvantages have been reviewed by Davis (1968). In the time available it was possible to test only a few of the cleaning and disinfecting agents commercially available, but at least three of the five tested have been or are used by large retail food organizations.

The results from Part I of this paper were not altogether surprising. The use of a disinfectant solution alone (Figs 1 and 2) gave rather unsatisfactory results. The use of a detergent solution alone gave somewhat better results, no doubt due to the removal of many of the bacteria present by the physical action of the detergent. The combined anionic detergent/disinfectant (20 p.p.m. available chlorine) solutions applied with clean cloths or disposable paper towels gave results only marginally better than detergent solutions alone. The use of disposable paper is to be preferred as the risk of cross-contamination will be greatly reduced (Gilbert, 1969). The use of a non-ionic detergent combined with a cationic disinfectant gave similar results to those obtained after using an anionic detergent solution. For the three items tested, slicing machine, carving knife and can-opener, the most satisfactory results were always obtained when an anionic detergent solution and a disinfectant solution (hypochlorite, 200 p.p.m. available chlorine) were used in a two-step procedure.

The use of the two vegetable oils for cleaning the slicing machine blade gave unsatisfactory results (Table 2) and this method of cleaning cannot be recommended.

The effectiveness of any cleaning method depends not only on the cleaning agent used but also on its being used regularly and intelligently. Some examples of cross-contamination by dirty equipment have been described previously (Gilbert, 1969) and so it is also important that the need for regular and effective cleaning should be understood.

The detection and enumeration of micro-organisms on equipment, utensils and working surfaces has attracted the attention of many microbiologists in recent years. The information so gained has been useful in comparative studies of microbiological cleanliness, e.g. for assessing cleaning procedures and for distinguishing 'cleaner' from 'dirtier' objects or areas. Numerous papers from the milk and food industries, from hospitals, and more recently from the aerospace industry have been published: general information on the various techniques available has been given by Walter (1955), Fincher (1965), Favero *et al.* (1968) and Schaeffer (1968). Many investigators have carried out comparative tests on two or more of the methods for surface sampling; the reports of Angelotti, Foter, Busch & Lewis (1958) and Mossel, Kampelmacher & van Noorle Jansen (1966) are noteworthy.

Mossel *et al.* (1966) reported that for counts of *Enterobacteriaceae* on wooden surfaces, a swab-rinse technique with calcium alginate swabs gave results greater by a factor of about 10 than those given by an agar sausage technique; the recovery medium for both techniques was crystal violet neutral red bile glucose agar. Riddle (1967) reported a geometric mean figure of 18 (range 3-286) for this factor when a swab-rinse technique with cotton wool swabs and an agar sausage technique were used for comparative counts on carcasses in bacon factories. The explanation for the observed differences between counts given in the present study by the two techniques is three-fold. (*a*) Compared to the agar sausage technique, the swab-rinse technique is more likely to receive bacteria lodged in microscopic fissures, surface irregularities or deposited on surfaces in dried films. (*b*) Compared to the agar sausage technique, the swab-rinse technique is likely to break up clumps or chains of bacteria when the rinse solution is shaken to dissolve the swabs. It is therefore more likely to measure individual bacterial cells/area tested than the agar sausage technique which provides a mirror image picture of the distribution of bacteria whether they are clumped or not. (*c*) Blood agar used as a recovery medium from the swab-rinse dilutions is a better non-selective growth medium than the plate count and MacConkey agars used for the agar sausages.

The agar sausage technique has been recommended for investigations by Public Health Inspectors (Greig, 1966; Eade, 1968) and as a routine control method for use in the food industry (Goldenberg & Relf, 1967; Riddle, 1967). A comprehensive list of references to uses of the technique has been given by Bridson (1969). It is a valuable visual-aid for the instruction of food handlers since it provides a simple pictorial demonstration of the presence of bacteria on a wide variety of surfaces and might encourage the more efficient cleaning of equipment. The simplicity, speed and economy of the technique together with its applicability in numerous situations make it a most useful routine control method for the relative day-to-day sampling of surfaces. The relatively low recovery rates given by this method in comparison with other techniques must be recognized and practical experience in the interpretations of results is necessary.

It is a pleasure to record my thanks to Dr Betty C. Hobbs and Dr J. C. Kelsey for their advice and constructive criticisms. I am also very grateful to Mr R. T. Govett, Senior Public Health Inspector of the London Borough of Harrow for his helpful and practical assistance. Such a project would not have been possible without the full co-operation of the directors, management and staff of the two self-service stores.

REFERENCES

Angelotti, R., Foter, M. J., Busch, K. A. & Lewis, K. H. (1958). A comparative evaluation of methods for determining the bacterial contamination of surfaces. *Food Research* **23**, 175.

Bridson, E. Y. (1969). Isolation of surface micro-organisms with the agar slice technique (agar sausage). In *Isolation Methods for Microbiologists*. Eds. D. A. Shapton and G. W. Gould. London: Academic Press.

Brookes, F. C. & Fennell, H. (1952). A powder-dusting test for the control of dish-washing and the comparison of detergents. *Monthly Bulletin of the Ministry of Health and Public Health Laboratory Service* **11**, 264.

CLEGG, L. F. L. (1955). Laboratory and field evaluation of chemical sterilizers for dairy farms. *Journal of Applied Bacteriology* **18**, 358.

COUSINS, C. M., HOY, W. A. & CLEGG, L. F. L. (1960). The evaluation of surface active disinfectants for use in milk production. *Journal of Applied Bacteriology* **23**, 359.

DAVIS, J. G. (1968). Chemical sterilization. In *Progress in Industrial Microbiology* **8**, Ed. D. J. D. Hockenhull. London: J. & A. Churchill Ltd.

EADE, S. A. (1968). An investigation into the efficiency of cleansing processes used in kitchens. *Public Health Inspector* **76**, 407.

FAVERO, M. S., MCDADE, J. J., ROBERTSEN, J. A., HOFFMAN, R. K. & EDWARDS, R. W. (1968). Microbiological sampling of surfaces. *Journal of Applied Bacteriology* **31**, 336.

FINCHER, E. L. (1965). Surface sampling for bacteria. Reprint no. 59, Biophysics Section, National Communicable Disease Center, Atlanta, Georgia, U.S.A.

FOOD HYGIENE CODE OF PRACTICE, no. 8 (1969). *Hygiene in the Meat Trades*. London: H.M.S.O.

GILBERT, R. J. (1969). Cross-contamination by cooked-meat slicing machines and cleaning cloths. *Journal of Hygiene* **67**, 249.

GILBERT, R. J. & MAURER, I. M. (1968). The hygiene of slicing machines, carving knives and can-openers. *Journal of Hygiene* **66**, 439.

GOLDENBERG, N. & RELF, C. J. (1967). Use of disinfectants in the food industry. *Journal of Applied Bacteriology* **30**, 141.

GREIG, J. R. (1966). An improved method of surface bacteriological sampling in food premises. *Public Health Inspector* **75**, 170.

HIGGINS, M. (1950). A comparison of the recovery rates of organisms from cotton-wool and calcium alginate swabs. *Monthly Bulletin of the Ministry of Health and Public Health Laboratory Service* **9**, 50.

HOBBS, B. C., EMBERLEY, N., PRYOR, H. M. & SMITH, M. E. (1960). The assessment of the activities of surface active agents for use in the catering industry. *Journal of Applied Bacteriology* **23**, 350.

HOY, W. A. & CLEGG, L. F. L. (1953). Testing farm detergent-sterilizers using soiled ten gallon cans. *Proceedings of the Society for Applied Bacteriology* **16**, i.

LISBOA, N. P. (1959). A tube test for evaluating agents possessing both detergent and sterilizing properties. *Proceedings of the* 15th *International Dairy Congress, London* **3**, 1816.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *Journal of Hygiene* **38**, 732.

MOSSEL, D. A., KAMPELMACHER, E. H. & VAN NOORLE JANSEN, L. M. (1966). Verification of adequate sanitation of wooden surfaces used in meat and poultry processing. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene*, I. Abt. orig. **201**, 91.

REPORT (1964). *The Aberdeen Typhoid Outbreak*, 1964. Edinburgh: H.M.S.O.

RIDDLE, W. (1967). The agar sausage technique and its use as a routine bacteriological control measure in food factories. *Technical Circular no.* 372, *British Food Manufacturing Industries Research Association*, Leatherhead, Surrey.

SCHAEFFER, R. L. (1968). Practical aspects of surface sampling. *Hospitals* **42**, 94.

TEN CATE, L. (1963). Eine einfache und schnelle bakteriologische Betriebskontrolle in Fleisch verarbeitenden Betrieben mittels Agar-Würsten in Rilsan-Kunstdarm. *Fleischwirtschaft* **15**, 483.

TEN CATE, L. (1965). A note on a simple method of bacteriological sampling by means of agar sausages. *Journal of Applied Bacteriology* **28**, 221.

WALTER, W. G. (1955). Symposium on methods for determining bacterial contamination on surfaces. *Bacteriological Reviews* **19**, 284.

J. Hyg., Camb. (1970), **68**, 233
Printed in Great Britain

The production of neutralizing activity in serum and nasal secretion following immunization with influenza B virus

By JEAN C. DOWNIE and C. H. STUART-HARRIS
The Virus Research Laboratory of the University of Sheffield, Lodge Moor Hospital, Sheffield

(*Received* 2 *January* 1970)

SUMMARY

Trials were made in volunteers in 1967 and 1968 of various virus vaccines against influenza virus B. Sera and serially collected nasal washings before and after immunization were tested respectively for haemagglutination-inhibiting and tissue culture virus-neutralizing antibodies to the same strain of influenza B/Eng/65 virus as that used in the vaccines. Infection, as determined by recovery of virus and serological changes following intranasal instillation of attenuated live virus, was accompanied by the subsequent appearance of neutralizing antibodies in nasal secretion. Inactivated vaccine subcutaneously did not evoke nasal antibody formation in 1967 but did so in 1968.

In 1968 intranasal challenge of the volunteers with the attenuated virus 1 month after immunization demonstrated a correlation of susceptibility or resistance to infection with nasal and serum antibodies. Resistance appeared to depend either on a high level of serum antibodies or nasal antibodies, or both.

INTRODUCTION

In contrast to many other virus infections of man, the protection resulting from a single attack of influenza virus infection in the adult has long been believed to be temporary. Nevertheless remarkably few data exist concerning the duration of immunity following proven influenza virus infection. Though Sigel, Kitts, Light & Henle (1950) reported recurrent infections in 1949 among schoolboys affected in 1947 by the same influenza virus A1, such a brief duration of immunity seems exceptional. J. Fry (personal communication) indeed believes from observations in his own practice that relatively few adults affected by Asian influenza in 1957 were again affected clinically during the recurrent outbreaks of 1959, 1961 and 1964.

Because of the difficulty of judging influenzal immunity after natural infections, the challenging effect of deliberate inoculation with living virus has been studied in volunteers. Thus more than 20 years ago laboratory-cultivated influenza viruses A and B were used to challenge the protection induced by subcutaneous immunization (Henle, Henle & Stokes, 1943; Francis, Salk, Pearson & Brown, 1945; Salk, Pearson, Brown & Francis, 1945).

More recently viruses modified by artificial cultivation in hens' eggs or tissue cultures and attenuated in virulence for man have been used as vaccines and also

to test immunity (McDonald, Zuckerman, Beare & Tyrrell, 1962; Beare *et al.* 1967; Beare, Bynoe & Tyrrell, 1968; Beare *et al.* 1969). As these viruses provoke little or no clinical reaction, resistance to their infection has been assessed by attempted reisolation of virus from the throat and antibody responses in the serum. McDonald *et al.* (1962), using the attenuated IKSHA A2 virus in volunteers, showed that intranasal inoculation of living virus was less likely to result in infection in persons whose sera contained demonstrable neutralizing antibodies than in those without such antibodies. Nevertheless, these same experiments showed a lack of precise correlation between serum antibody titres and resistance to challenge infection such as was observed 30 years ago in relation to clinical influenza in the field (Hoyle & Fairbrother, 1937; Stuart-Harris, Andrewes & Smith, 1938; Rickard, Horsfall, Hirst & Lennette, 1941). Some persons with moderate titres of serum antibody were infected and conversely some persons without such antibodies resisted infection (Andrews *et al.* 1966; Beare *et al.* 1967).

Nasal antibody inhibitory to influenza virus A was first demonstrated by Francis (1940) and was found to increase after infection by Francis & Brightman (1941). Its increase following subcutaneous influenzal vaccination was suggested by Francis, Pearson, Sullivan & Brown (1943) and Mulder, Brans & Hers (1952). However this early work involved the collection of nasal secretion by a method which may have led to transudation of plasma or blood and immunological tests on nasal secretion require that secretion should be collected by simple wash-out with saline. By this method Alford, Rossen, Butler & Kasel (1967), and Mann *et al.* (1968) studied nasal secretions from volunteers either infected with living A2 virus or inoculated subcutaneously with inactivated virus vaccine. Little neutralizing antibody was found in the nasal secretion before these procedures but afterwards antibody titres rose in both groups, though to a much greater extent in those receiving live virus than in those inoculated with vaccine.

Waldman *et al.* (1968) used the respiratory route to introduce inactivated virus into the body by means of a coarse intranasal spray. Serum antibody responses were less than after subcutaneous inoculation but increases were found in the nasal antibody titre more often after intranasal application of vaccine. Kasel *et al.* (1969) have confirmed that a better nasal antibody response occurs after intranasal than after subcutaneous inactivated polyvalent influenza vaccine.

The present study was designed to compare the changes in the nasal secretions of volunteers following immunization with various influenza B vaccines and to attempt to correlate antibodies in the serum or nasal secretion with resistance to a test infection.

METHODS

Virus vaccines

Influenza B/Eng/13/65 virus (Beare *et al.* 1968), which had been passaged six times in leucosis-free eggs, was kindly supplied to Dr D. C. Breeze, Evans Medical Limited, Speke. This virus had a titre of $10^{9\cdot4}$ egg infective doses (EID 50) per ml. It was used intranasally in a volume of 1·0 ml. (0·5 ml. into each nostril) diluted in balanced Hanks's saline to contain $10^{5\cdot4}$ EID 50 per ml.

Formalin-killed vaccines were prepared from the same virus B strain or an A2 virus (A2/Eng/1/61) by Evans Medical Limited, and were given by deep subcutaneous inoculation in 1·0 ml. doses containing 5500 haemagglutinating units.

Nasal specimens

Nasal washings were collected by the volunteers themselves before and during the period of observation of each trial. Specimens were obtained by instilling approximately 2 ml. of sterile phosphate buffered saline (pH 7·2) into each nostril of the subject with the head held well back and the glottis closed. The fluid was then forcibly expelled into a sterile Petri dish. This procedure was repeated four or five times. In practice, the average specimen obtained was 9 ml. of nasal secretion. The washings were shaken vigorously with glass beads, centrifuged at 3000 rev./min. for 15 min. and the supernatant fluid stored at −20°C. The specimens were tested for the presence of haemoglobin by the guaiac test and positive specimens were discarded.

Serological methods

Tissue culture haemadsorption test. The nasal secretions were first heated at 56°C. for 30 min. In the test undiluted secretion was mixed with an equal volume of 10 or 100 tissue culture infective doses of influenza virus B (TCID 50) as determined by haemadsorption and held at 4°C. for 1 hr. Each of four tubes of Patas monkey kidney cultures was inoculated with 0·2 ml. of the mixture, incubated for 3 days at 35–36°C. and examined for haemadsorption with 0·5% guinea-pig cells. Antibody was considered to have been present if there was more than a 75% reduction in haemadsorption in comparison with control cultures of virus only.

Haemagglutination inhibition (H.I.) test. Serum specimens were diluted 1/6 with cholera filtrate (Philips Duphar, Holland) and incubated overnight at 37°C. Enzyme was then inactivated by incubation at 56°C. for 60 min. before testing. H.I. tests were carried out in WHO plastic trays using 0·2 ml. volumes of serum and an equal volume containing eight haemagglutinating units of virus. The virus–serum mixtures were incubated at room temperature for 60 min. before adding 0·2 ml. of 0·5% fowl red blood cells.

Complement fixation (C.F.) test. The sera were tested for complement-fixing antibodies by standard methods using 2 MHD of complement and overnight fixation at 4°C. Before testing they were inactivated at 56°C. for 30 min. (Bradstreet & Taylor, 1962). The antigen used in the complement fixation test was kindly supplied by Dr C. M. P. Bradstreet, Public Health Laboratory Service, Colindale.

Virus isolation

Throat swabs were taken from volunteers given the live vaccine and from all the volunteers after challenge on the 2nd and 3rd day after inoculation. The swabs were placed in transport medium containing 2·5% bovine serum albumin and polymyxin 125 units/ml. and stored at −70°C. Specimens for virus isolation were inoculated into the amniotic sac of 10-day-old embryonated hens' eggs, and into

Rhesus monkey kidney tissue culture cells. Eggs were harvested after 3 days incubation at 33°C. and the amniotic fluids were tested for virus haemagglutinins. Monkey kidney cells were tested for haemadsorption after 3–5 days incubation. Negative specimens were passed three times in eggs and twice in tissue culture before being discarded.

Tissue cell cultures

Patas monkey kidney cells were obtained from Burroughs Wellcome Limited, Beckenham, Kent, and Rhesus monkey kidney cells were supplied by the Biological Standards Division of the National Institute for Medical Research. These cells were grown in mixture '199' fluid with 5% calf serum at an initial concentration of 8×10^5 viable cells/ml., and confluent cell cultures were maintained in mixture '199' without serum.

THE VACCINATION TRIALS

In 1967 fifteen medical students aged 21 to 23 volunteered to participate in a comparison of inactivated and live virus B vaccines. Eight students were inoculated intranasally with live influenza B virus vaccine and seven were given inactivated vaccine subcutaneously. Nasal secretions were collected before and for 21 days after immunization. Serum was collected from each student before immunization, and at 1, 3 and 23 weeks after immunization. Stored specimens (−20°C.) were tested for H.I. and C.F. antibodies.

In 1968 a further study of similar vaccines in 39 students, who had not taken part in the previous trial, was completed by a challenge inoculation of the live vaccine virus B intranasally. There were four vaccine groups. Eleven students received intranasal live B/Eng/13/65 virus in the same dose and of the same batch as that used in 1967 and 11 received inactivated B vaccine prepared from the same strain of virus and given as a coarse intranasal spray in a 1 ml. dose. Eight students received the inactivated B vaccine subcutaneously and nine received subcutaneously an inactivated vaccine prepared from an A2 virus. The latter served as controls to the students receiving B vaccines. One month after immunization all volunteers received a challenge intranasal inoculation of 1·0 ml. of the live B virus vaccine diluted in Hanks's saline to contain $10^{6\cdot4}$ egg infective doses (EID 50). Nasal washings were obtained before and at intervals after immunization and challenge. Sera were collected before immunization, 3 weeks after immunization, and 3 weeks after challenge. They were stored at −20°C. and tested as above.

The results of immunization

Table 1 shows that in the 1967 trial six of eight volunteers inoculated with live virus B became infected, as shown by a fourfold or greater rise in antibody. Virus was recovered from four of the six persons. Neutralizing antibody was not detected in nasal secretion before immunization in any person but in those who became infected with live virus it was detected from the 8th to the 18th day after inoculation. Five of the seven volunteers given subcutaneous vaccine developed

a fourfold or greater rise in serum H.I. antibodies yet neutralizing antibody in nasal secretions was not demonstrated during the 21 days of observation.

Table 2 shows the results obtained in 1968, which differed from those in 1967. A fourfold or greater rise in serum H.I. antibodies to influenza B occurred in seven of eight persons receiving inactivated virus B vaccine subcutaneously, in

Table 1. *Serum and nasal antibody responses in volunteers immunized with live and inactivated influenza* B/Eng/13/65 *virus vaccines: 1967 study*

Immunization group	No. of volunteers	Evidence of immunization		No. showing nasal antibodies when tested against 10 TCID 50	
		Virus isolation	No. showing fourfold rise in HI antibodies	Before immunization	After immunization
B/Eng/13/65. Live I.N.	8	4	6	0	6*
B/Eng/13/65. Inactivated S.C.	7	—	5	0	0

* Five of these specimens neutralized 100 TCID 50 of virus.

Table 2. *Serum and nasal antibody responses in volunteers immunized with live and inactivated influenza virus vaccines: 1968 study*

Immunization group	No. of volunteers	No. showing fourfold rise in HI antibodies	No. showing nasal antibodies before and after immunization when tested against			
			10 TCID 50		100 TCID 50	
			Before	After	Before	After
B/Eng/13/65. Live I.N.	11*	1	2	7	1	4
B/Eng/13/65. Inactivated I.N.	11	1	1	8	0	2
B/Eng/13/65. Inactivated S.C.	8	7	3	8	1	4
A2/Eng/1/61. Inactivated S.C.	9	0	2	6	0	1

* No virus was reisolated from any person.

one of each of the groups of 11 persons receiving live or inactivated influenza B virus intranasally and in none of the nine persons who received A2 vaccine subcutaneously.

Neutralizing antibodies to virus B were found in the nasal secretions of eight of the 39 volunteers in the various groups before immunization and in 29 of the volunteers on one or more occasions during the 28 days after immunization. Washings which neutralized 100 TCID 50 were found in only two persons before and in 11 after including four of 11 persons given live virus intranasally and four of eight persons receiving inactivated B vaccine subcutaneously. Eight of 11

persons receiving inactivated vaccine intranasally had nasal washings after immunization with inhibitory effects upon 10 TCID 50 and two inhibited 100 TCID 50 of virus.

Two of the nine persons immunized with A2 vaccine had nasal washings inhibitory to 10 but not to 100 TCID 50 before immunization. After immunization, which had failed to increase serum antibodies to virus B, six of the nine persons had nasal washings inhibitory to 10 TCID 50 of virus B but only a single specimen from one of these also inhibited 100 doses of virus. This unexpected result could not be readily explained unless virus B had spread from one of the volunteers given live virus intranasally. As none of these 11 persons yielded viruses by direct test of throat swabs this possibility seemed less likely than others such as a nasal non-specific inhibitor other than antibody with weak antiviral properties or a non-specific boosting of local antibody formation. Inhibition of 100 TCID 50 of virus by nasal washings was thought to be unlikely to be due to other than neutralizing antibody and such a property was generally found in consecutive nasal washings from the persons immunized with various B vaccines and not in just a single specimen as in the case of the A2 vaccinated group.

The effects of challenge of the 1968 *volunteers*

Table 3 shows the results of the challenge inoculation of all the volunteers with live B virus intranasally on the 28th day after immunization and their nasal antibody status at this time. The dose of virus was increased to $10^{6.4}$ EID 50 because

Table 3. *Immune status of immunized volunteers before and after challenge with live influenza* B/Eng/13/64 *virus: 1968 study*

Immunization group	No. of volunteers	No. showing neutralizing activity in nasal secretion within 16 days before challenge		No. showing evidence of infection by challenge virus by	
		Against 10 TCID 50 virus	Against 100 TCID50 virus	Virus isolation	Fourfold rise in HI antibodies
B/Eng/13/65. Live I.N.	11	5	4	1	2
B/Eng/13/65. Inactivated I.N.	11	7	2	1	2
B/Eng/13/65. Inactivated S.C.	8	8	3	1	0*
A2/Eng/1/61. Inactivated S.C.	9	6	0	0	3†

* Rise of C.F. antibodies in one person.
† Three-fold rise of H.I. antibody in one other person.

the previous dose of $10^{5.4}$ EID 50 given to volunteers had failed to yield virus from throat swabs. Nine of the 39 students experienced a common cold-like illness after inoculation with the larger dose of virus. Virus was reisolated from three persons, each of whom developed a fourfold or greater rise in serum antibodies by either

Table 4. *Serum titres and neutralizing activity in nasal secretion of susceptible volunteers before challenge with live influenza* B/Eng/13/65 *virus:* 1968 *study*

			Serum and nasal secretion antibody within 16 days prior to challenge			Evidence of infection with challenge virus	
				Neutralizing activity nasal secretions			
Immunization group	Total no.	Susceptible persons	Serum antibodies (H.I.)	10 TCID 50 virus	100 TCID 50 virus	Virus isolation	Rise in serum H.I. antibodies (4 × or more)
B/Eng/13/65.	11	J.	1/12	+	+	−	+
Live I.N.		R.	1/9	0	0	+	+
B/Eng/13/65.	11	P.	< 1/6	+	0	−	+
Inactivated I.N.		L.	< 1/6	+	0	+	+
B/Eng/13/65.	8	M.	1/36	+	0	+	0*
Inactivated S.C.							
A2/Eng/1/61.	9	L.	1/9	0	0	−	+
Inactivated S.C.		I.	1/9	+	0	−	+
		C.	< 1/6	+	0	−	+
		E.	1/18	+	0	−	+ †

* Rise of C.F. antibodies × 4.
† Rise of H.I. antibodies × 3.

Table 5. *Serum titres and neutralizing activity in nasal secretions of volunteers to infection by challenge with live influenza* B/Eng/13/65 *virus: 1968 study*

Immunization group	Total no. of volunteers	No. resistant to infection	Serum antibodies (H.I.) within 16 days prior to challenge			Neutralizing activity nasal secretions			
						Within 16 days prior to challenge		After challenge	
			< 1/6	1/6–1/18	> 1/24	10 TCID 50 virus	100 TCID 50 virus	10 TCID 50 virus	100 TCID 50 virus
B/Eng/13/65. Live I.N.	11	9	2	1	6	4	3	7	4
B/Eng/13/65. Inactivated I.N.	11	9	2	5	2	5	2	8	3
B/Eng/13/65. Inactivated S.C.	8	7	0	1	6	7	3	7	5
A2/Eng/1/61. Inactivated S.C.	9	5	1	2	2	3	0	4	2

H. I. or C.F. test. Five more developed a fourfold increase in H.I. antibodies, and one showed a threefold antibody rise. Four volunteers with serological evidence of infection belonged to the control group originally given A2 vaccine. Altogether nine infections were judged to have occurred among the 39 immunized persons (Table 4).

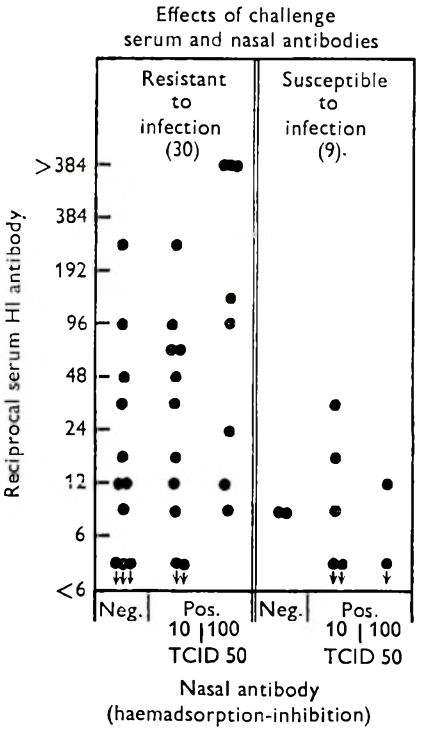

Fig. 1. Diagrammatic representation of serum and nasal antibodies in immunized volunteers before attempted infection with intranasal live B/Eng/65 virus.

Table 4 shows the H.I. serum titres just before challenge and also the presence or absence of neutralizing antibody in nasal secretions collected during the previous 16 days before challenge for each of the nine volunteers. Three persons had no detectable serum antibodies at a 1/6 dilution and only one had a serum titre in excess of 1/24. Neutralizing antibody was not detected in nasal secretions from two of the volunteers but was detected in seven against 10 TCID 50 of virus and in one against 100 TCID 50 as well.

The results in those who resisted challenge by neither yielding virus from throat swabs nor developing a fourfold or greater increase in serum antibodies are shown in Table 5 grouped together by method of immunization. Out of 30 resistant volunteers, 16 (53 %) possessed serum H.I. antibody titres of or in excess of 1/24 and only five failed to inhibit haemagglutination at 1/6 dilution of serum. Inhibitory activity was present against 10 TCID 50 of virus in the nasal secretion before challenge in 19 persons and was active against 100 TCID 50 of virus in eight instances. Serum H.I. antibody was present in a titre of or in excess of 1/24 in 12 persons in whom antibody was also present in nasal secretion. Of these 12 persons,

the nasal washings from six inhibited 100 TCID 50 of virus, and from six more 10 TCID 50. Among the 14 whose serum titre was less than 1/24, neutralizing activity was present in the nasal secretion in seven but was only active against 100 TCID 50 in two persons.

These results are presented diagrammatically in Fig. 1, which compares serum antibodies and nasal washings inhibitory to virus at the time of the challenge infection in both resistant and susceptible volunteers. Resistance to infection was correlated to some extent with the titre of serum H.I. antibodies in that those with titres of 1/24 or more escaped infection except in one instance. Resistance was exhibited by 11 volunteers in whom nasal antibodies were not demonstrated. Four had high serum titres (> 1/24) but seven did not and in these some other factor may have been responsible for the resistance. Including those with nasal inhibition of only 10 TCID 50 and those inhibiting 100 TCID 50 of virus, there were seven of 26 persons with nasal inhibition who contracted infection but all but one of these had low serum antibody levels less than 1/24. Most of the 19 other persons, who resisted infection, had demonstrable serum antibodies as well as nasal inhibitory substances but two had none.

DISCUSSION

It has been known for 25 years that inactivated influenza virus vaccines will confer a significant degree of immunity against influenza in the field (Report of the Medical Research Council Committee, 1958; Davenport, 1961). This immunity has traditionally been believed to be mediated by antibody formation and the recent experimental work on small numbers of persons confirms that these antibodies appear after subcutaneous inoculation in both serum and nasal secretions (Mann *et al.* 1968; Kasel *et al.* 1969).

Waldman *et al.* (1968), using an inactivated A2 virus, and Kasel *et al.* (1969), using a polyvalent vaccine, found that the intranasal route was better for inducing neutralizing antibody in nasal secretions than was the subcutaneous route. However, in 1968 we found that inactivated influenza B virus gave a better neutralizing antibody response in nasal secretions after subcutaneous inoculation than after intranasal immunization. The poor response in nasal secretions after intranasal vaccine may have been due to the low dose employed, since the volunteers were not given a booster inoculation.

The present study has emphasized the great difficulty in obtaining consistent results in volunteers perhaps because of changes in the immune status of the population resulting from natural exposure to infection. Thus the ready demonstration of nasal antibodies before immunization in the volunteers in 1968 compared with its absence from others in 1967 is strongly suggestive of recent exposure to influenza B virus. Moreover, the results of attempted infection with attenuated virus are also consistent with exposure to influenza B virus in the preceding months of 1968, and indeed there was a ten-fold increase in reported illnesses due to influenza B virus during the period from mid-March to mid-April 1968.

The evidence collected by workers with viruses such as parainfluenza Type I (Smith, Purcell, Bellanti & Chanock, 1966) and rhinoviruses (Cate *et al.* 1966;

Fleet, Couch, Cate & Knight, 1965) have shown that both serum and nasal antibodies are required to confer resistance to direct challenge by intranasal virus. The experiments made in Chicago with parainfluenza Type 2 virus by Tremonti, Lin & Jackson (1968) also suggest a synergistic effect of nasal and serum antibody. Our own work with influenza B supports these findings in that resistance to infection sometimes appeared to depend upon serum antibodies only but more often upon a combination of serum and nasal antibodies. Persons susceptible to infection mostly possessed weak or no inhibitory activity in nasal washings.

Infection might also have been resisted on some basis other than neutralizing antibody since three of the volunteers who were not susceptible to the challenge dose of virus lacked neutralizing antibody in both nasal secretions and serum. Gresser & Dull in 1964 described an interferon-like inhibitor in pharyngeal washings from patients with clinical influenza. In 1965 Jao, Wheelock & Jackson detected an interferon in nasal washings and serum of volunteers challenged with a live influenza A2 virus. It has also been observed that after infection with common cold-like viruses there is an increased resistance to infection by other viruses (Lidwell & Williams, 1961; Fleet *et al.* 1965). Finally, antibody to influenzal neuraminidase, although not preventing the initiation of infection, may be involved in limiting the extent of infection in the host. Schulman, Khakpour & Kilbourne (1968) have found that neuraminidase-inhibiting antibody increased the survival of mice when challenged with influenza virus.

This work could not have been performed without the asisstance of Dr D. C. Breeze, of Evans Medical Ltd., who provided the various vaccines. We should like to thank all the volunteers for their patient co-operation, and Mrs K. Spinks for her excellent technical assistance. One of us (C. H. S.-H.) is in receipt of a grant from the Medical Research Council.

REFERENCES

Alford, R. H., Rossen, R. D., Butler, W. T. & Kasel, J. A. (1967). Neutralising and haemagglutination-inhibiting activity of nasal secretions following experimental human infection with A2 influenza virus. *Journal of Immunology* **98**, 724.

Andrews, B. E., Beare, A. S., McDonald, J. C., Zuckerman, A. J. & Tyrrell, D. A. J. (1966). Further trials of live influenza vaccine. *British Medical Journal* i, 637.

Beare, A. S., Bynoe, M. L. & Tyrrell, D. A. J. (1968). Investigation into the attenuation of influenza viruses by serial passage. *British Medical Journal* ii, 482.

Beare, A. S., Tyrrell, D. A. J., Hobson, D., Howells, C. H. L., Pereira, M. S., Pollock, T. M. & Tyler, L. E. (1969). Live Influenza B vaccine in volunteers. *Journal of Hygiene* **67**, 1.

Beare, A. S., Tyrrell, D. A. J., McDonald, J. C., Pollock, T. M., Taylor, C. E. D., Howells, C. H. L. & Tyler, L. E. (1967). Reactions and antibody response to live influenza vaccine prepared from the Iksha (A2) strain. *Journal of Hygiene* **65**, 245.

Bradstreet, C. M. P. & Taylor, C. E. D. (1962). Technique of complement-fixation test applicable to the diagnosis of virus diseases. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **21**, 96.

Cate, T. R., Rossen, R. D., Douglas, R. G., Jr, Butler, W. T. & Couch, R. B. (1966). The role of nasal secretion and serum antibody in the rhinovirus common cold. *American Journal of Epidemiology* **84**, 352.

Davenport, F. M. (1961). Inactivated influenza virus vaccines. *American Review of Respiratory Diseases* **83**, 146.

FLEET, W. F., COUCH, R. B., CATE, T. R. & KNIGHT, V. (1965). Homologous and heterologous resistance to rhinovirus common cold. *American Journal of Epidemiology* **82**, 185.

FRANCIS, T., Jr (1940). The inactivation of epidemic influenza virus by nasal secretions of human individuals. *Science, New York* **91**, 198.

FRANCIS, T., Jr & BRIGHTMAN, I. J. (1941). Virus-inactivating capacity of nasal secretions in the acute and convalescent stages of influenza. *Proceedings of the Society for Experimental Biology and Medicine* **48**, 116.

FRANCIS, T., Jr, PEARSON, H. E., SULLIVAN, E. R. & BROWN, P. M. (1943). The effect of subcutaneous vaccination with influenza virus upon the virus-inactivating capacity of nasal secretions. *American Journal of Hygiene* **37**, 294.

FRANCIS, T., Jr, SALK, J. E., PEARSON, H. E. & BROWN, P. N. (1945). The protective effect of vaccination against induced influenza A. *Journal of Clinical Investigation* **24**, 536.

GRESSER, I. & DULL, H. B. (1964). A virus inhibitor in pharyngeal washings from patients with influenza. *Proceedings of the Society for Experimental Biology and Medicine* **115**, 192.

HENLE, W., HENLE, G. & STOKES, J., Jr (1943). Demonstration of the efficacy of vaccination against influenza type A by experimental infection of human beings. *Journal of Immunology* **46**, 163.

HOYLE, L. & FAIRBROTHER, R. W. (1937). Isolation of the influenza virus and the relation of antibodies to infection and immunity. *British Medical Journal* i, 655.

JAO, R. L., WHEELOCK, E. F. & JACKSON, G. G. (1965). Interferon study in volunteers infected with Asian influenza. *Journal of Clinical Investigation* **44**, 1062.

KASEL, J. A., HUME, E. B., FULK, R. V., TOGO, T., HUBER, M. & HORNICK, R. B. (1969). Antibody responses in nasal secretions and serum of elderly persons following local or parenteral administration of inactivated influenza virus vaccine. *Journal of Immunology* **102**, 555.

LIDWELL, O. M. & WILLIAMS, R. E. O. (1961). The epidemiology of the common cold. II. Cross-infection and immunity. *Journal of Hygiene* **59**, 321.

MANN, J. J., WALDMAN, R. H., TOGO, Y., HEINER, G. G., DAWKINS, A. T. & KASEL, J. A. (1968). Antibody response in respiratory secretions of volunteers given live and dead influenza virus. *Journal of Immunology* **100**, 726.

MCDONALD, J. C., ZUCKERMAN, A. J., BEARE, A. S. & TYRRELL, D. A. J. (1962). Trials of live influenza vaccine in the Royal Air Force. *British Medical Journal* i, 1036.

MULDER, J., BRANS, L. M. & HERS, J. F. Ph. (1952). Studies on the increase of antibodies in nasal secretions of human beings after intracutaneous vaccination with influenza virus with the aid of the haemagglutination-inhibition technique. *Antonie van Leeuwenhoek* **18**, 131.

REPORT OF COMMITTEE ON CLINICAL TRIALS OF INFLUENZA VACCINE (M.R.C.) (1958). Trials of Asian influenza vaccine. *British Medical Journal* i, 415.

RICKARD, E. R., HORSFALL, F. L., HIRST, G. K. & LENNETTE, E. H. (1941). The correlation between neutralizing antibodies in serum against influenza viruses and susceptibility to influenza in man. *Public Health Reports, Washington* **56**, 1819.

SALK, J. E., PEARSON, H. E., BROWN, P. N. & FRANCIS, T., Jr (1945). Protective effect of vaccination against induced influenza B. *Journal of Clinical Investigation* **24**, 547.

SCHULMAN, J. L., KHAKPOUR, M. & KILBOURNE, E. D. (1968). Protective effects of specific immunity to viral neuraminidase on influenza virus infection of mice. *Journal of Virology* **2**, 778.

SIGEL, M. M., KITTS, A. W., LIGHT, A. B. & HENLE, W. (1950). The recurrence of influenza A-prime in a boarding school after 2 years. *Journal of Immunology* **64**, 33.

SMITH, C. B., PURCELL, R. H., BELLANTI, J. A. & CHANOCK, R. M. (1966). Protective effect of antibody to parainfluenza type 1 virus. *New England Journal of Medicine* **275**, 1145.

STUART-HARRIS, C. H., ANDREWES, C. H. & SMITH, W. (1938). Study of epidemic influenza with special reference to the 1936–37 epidemic. *Medical Research Council. Special Report Series* No. 228.

TREMONTI, L. P., LIN, J-S.L. & JACKSON, G. G. (1968). Neutralising activity in nasal secretions and serum in resistance of volunteers to parainfluenza virus type 2. *Journal of Immunology* **101**, 572.

WALDMAN, R. H., KASEL, J. A., FULK, R. V., TOGO, Y., HORNICK, R. B., HEINER, G. G., DAWKINS, A. T., Jr & MANN, J. J. (1968). Influenza antibody in human respiratory secretions after subcutaneous or respiratory immunization with inactivated virus. *Nature, London* **218**, 594.

J. Hyg., Camb. (1970), **68**, 245
Printed in Great Britain

Comparative toxicity of various ozonized olefins to bacteria suspended in air

By F. A. DARK and T. NASH
Microbiological Research Establishment, Porton Down, Wilts.

(*Received* 9 *January* 1970)

SUMMARY

Air containing olefin vapour was treated with known amounts of ozone simulating natural concentrations. The bactericidal effect of the mixture was tested using microthreads sprayed with washed cultures of *Escherichia coli* var. *communis* or *Micrococcus albus*, aerosol strain. With 20 different olefins a wide range of activity was found, those in which the double bond formed part of a ring being the most bactericidal; petrol vapour was about as active as the average open-chain olefin. The two organisms behaved similarly at the experimental relative humidity of 80 %. The estimated amount of bactericidal substance present was only about one hundredth of that required to give the same kill with a 'conventional' air disinfectant; a simple physical explanation is proposed for this enhanced effect.

INTRODUCTION

Rural air often contains a bactericidal component, present in such low concentration that direct chemical identification has not yet been possible (Druett & May, 1968). For convenience it has been called the Open Air Factor (OAF) and indirect evidence suggests that it arises from chemical reaction between ozone and olefins (Druett & Packman, 1968; Druett & May, 1968). Olefins are present in the open air because of the widespread dissemination of petroleum products, while ozone is a natural constituent of clean air. OAF is presumed to form whenever such clean upper air is brought down by turbulence and mixes with polluted lower air. This paper describes laboratory experiments on the bactericidal properties of air initially containing about 1 part per million of olefin vapour and about 1 part per hundred million of ozone, the latter concentration being comparable to that found naturally.

EXPERIMENTAL

OAF was discovered using the microthread technique, in which bacteria are held on spider escape line wound across a metal frame (May & Druett, 1968). In this way organisms are recoverable after exposure to open air for any required length of time. The same technique was used in the present work, the bacteria being exposed to the atmosphere inside a closed box. Bacteria on threads do not behave exactly as though they were free-floating, but the differences are not important in the present investigation of the comparative bactericidal activity of different atmospheres.

Organisms

Two test organisms were used. The first (EC) was *Escherichia coli* commune (MRE strain 162), a robust organism used in most of the open-air tests already mentioned. The second (MA) was *Micrococcus albus*, aerosol strain (N.C.T.C. 7944), which does not form large clumps and had been used before in tests of air disinfectants (Nash, 1962). The marker organism (BG) was *Bacillus subtilis* var. *niger* spores, also as used in the earlier Porton tests. The use of BG as a marker has been thoroughly investigated (Anderson, 1966; May & Druett, 1968).

Preparation and assay of microthreads

Microthreads on frames were infected by loading into a tubular brass manifold down which could be passed a concentrated bacterial cloud from a Collison spray. The spray pot contained a distilled water suspension of 2–3 $\times 10^8$ EC or MA and 1–2 $\times 10^8$ BG per ml. The cloud was somewhat diluted by bleeding in dry air until the relative humidity (R.H.) was near that of the experiment, generally 80 %. For assay, groups of three frames were dropped into 12 ml. of phosphate buffered sucrose/alginate solution, and after dilution this was plated on agar containing hydrolysed casein as nutrient.

Exposure chamber

Bacteria on microthreads were exposed in a rectangular welded aluminium box 4 ft. high, 3 ft. wide and 2 ft. deep, of volume 670 l. A removable panel occupied part of the front face, leaving a rectangular hole 29 in. wide by 13 in. high. The box was stiffened by angle aluminium around this opening in order that the panel should fit well. Below it there was a row of regularly spaced circular holes 1 in. in diameter, each closed by a cork. A narrow metal tube projected centrally through each cork to a depth of 4 in. inside the box, for holding the frames. In this way any number of frames up to twelve could be exposed to the box atmosphere at the same time, as long as required, and then withdrawn and tested for viable bacteria.

At the side of the box there was a small ozone generator consisting of a cylindrical 2-l. tin containing five Philips OZ4 lamps connected in series with each other and with a 100 W. filament bulb outside as a ballast resistor. An electronic timer allowed mains voltage to be placed across bulb and lamps for accurate periods of 1, 2, 3, 4 or 5 sec. Oxygen from a cylinder could be passed through the tin and into the box, at such a rate that 95 % of the ozone was swept through in 25 sec., after the lamps had been switched off.

Olefin vapour was introduced through one of the 1 in. holes by means of a calibrated syringe. The air inside the box was stirred by a large slow-speed fan at the top; using a Kata thermometer it was found that the air velocity past the frames was 10 to 15 cm./sec. The humidity inside the box could be increased by hanging up a damp cloth, and removing it when experience indicated. Relative humidity inside the box was measured by a polymer-film resistor on a separate small removable panel at the side. After an experiment the box was ventilated by removing

the front panel and passing in a length of 6 in. trunking connected to the laboratory air-conditioning system.

Air quality

The concentration of ozone used in the experiments was no greater than is often found naturally in rural air. Such ozone was not removed by the laboratory air-conditioning system, to any great extent, so that it was essential to remove it in some way from the box air before an experiment. On the other hand, when the outside air was polluted, the ozone might well have been used up leaving an excess of pollutants capable of reacting with more ozone if it was added. In order to deal with both of these contingencies, every experiment was preceded by a clean-up, as follows.

Test procedure

The air in the box was humidified as described above, and the box closed. Ozone was then injected, using one or two 5 sec. bursts from the lamps, and allowed 20 min. to burn up any reactive hydrocarbons or nitric oxide present. The particular olefin under test was then added, and allowed to stand for another hour. This destroyed residual ozone and also allowed time for the decay of any bactericidal product. The experiment proper was then started by exposing a control set of three frames for 10 min., to the olefin vapour only. After this frames were exposed, also for 10 min. at a time, to the olefin vapour and also to ozone which was injected with mixing at the beginning of the exposure. Successive sets of frames were exposed to increasing amounts of ozone, by switching on the lamps for 2, 4 or twice 5 sec.; the olefin was however nearly always in vast excess and its concentration could be assumed constant. Ten minutes was allowed between exposures in order that the bactericidal effect of the previous 'shot' of ozone should have decayed. The procedure of roughly trebling concentrations of ozone in successive tests also ensured that carrying-over of this kind would be negligible.

Choice of ozone and olefin concentrations

The initial ozone concentration in the box during an experiment was estimated by two methods which gave good agreement. In the first, the whole output of the generator was taken through a solution of colorimetric reagent (Nash, 1967) giving the total dose. In the second, the ozone concentration in the box was estimated (in the absence of olefin) by using a Brewer bubbler and cell (Brewer & Milford, 1960). The actual concentrations of ozone used were known from earlier work to give reasonable kills. The olefin concentration was more or less dictated by the conditions of the experiment. In comparing the effects of exposure to different substances, it was desirable to keep the time of exposure constant. If the various olefins had all been used at some fixed concentration, the rate of generation of bactericidal product would have varied in a gross and arbitrary manner from substance to substance, because olefins differ widely in the rate at which they react with ozone. It was therefore decided to use each olefin at such a concentration that

the ozone half-life in it was always 5 min. In all the experiments, therefore, the concentration of bactericidal substance presumably rose to a maximum during the 10 min. exposure, and then fell away, in a similar manner for all the olefins.

Measurement of ozone–olefin reaction rates

Varying the olefin concentration to suit its reactivity seems a good way of obtaining meaningful comparative results, but it has to be established first that with all of the olefins the required concentration is sufficiently high for the reaction to be pseudounimolecular, and also that the olefin itself is not bactericidal at this concentration. The latter condition was established in the course of the work, through the control exposures, while the former was put on a sound basis by a long series of preliminary experiment on the decay of ozone in the presence of various concentrations of each olefin. A box atmosphere containing 3–5 parts per hundred million (pphm) of ozone was prepared, and a known volume of olefin vapour injected. The decay of the ozone was then followed on the above-mentioned Brewer instrument, and plotted logarithmically. A half-life was then calculated and the experiment repeated once or twice until the 5 min. half-life was bracketed.

With ethylene, propylene and the butenes, gas from a small cylinder was used. With the other olefins, which are liquids at room temperature, a known volume of saturated vapour was injected into the box from a graduated syringe holding excess liquid at the plunger end. Data on vapour pressures were obtained from the *Handbook of Chemistry and Physics* (40th ed.) and when these were not given the vapour pressure of the corresponding saturated hydrocarbon was used, with a small correction for the double bond. With anethole considerable extrapolation was required, owing to its exceptionally low vapour pressure.

Any errors regarding vapour pressure can always be corrected later, as the actual volume of vapour injected is known. As a precaution against the presence of more volatile and reactive impurities, the air in the syringe was blown out a few times before injection. With only one substance was there definite evidence of a change in the composition of the vapour during volatilization. This was cyclopentadiene (not reported in the next section), which gave a spuriously high kill from the first vapour fraction, probably cyclopentene. When this was blown off the kill was quite low.

RESULTS

When the ozone concentration was plotted against time it was found that the die-away in the absence of added olefin was quite slow, with a half-life of 4 hr. As it was intended to add sufficient olefin to reduce the half-life to 5 min., such background die-away could be ignored. In the presence of olefin, the die-away followed the unimolecular law quite well in most cases, but with the conjugated ethers (ethyl vinyl ether, butyl vinyl ether and dihydropyran) there was considerable 'tailing'. It was always possible to define a half-life for the purpose of the bactericidal experiments, but only when the die-away showed a good straight logarithmic plot was it possible to calculate reaction velocity constants. When this was done they were found to be in broad agreement with those available in the literature;

different workers by no means agreed among themselves, however (Leighton, 1961; Bufalini & Altshuller, 1965).

Olefin concentrations required to give an ozone half-life of 5 min. are listed in Table 1, and were calculated as described above; the actual volumes of vapour injected into the box are also given.

Table 1. *Concentrations in parts per million of olefins required to give an ozone half-life of 5 min., at 19–22°C.*

(Figures in parentheses are ml. of saturated vapour added to 670 litres reaction volume.)

Terminal olefins			Internal olefins		
Ethylene	70	(45)	*Cis* 2-butene	0·7	(0·45)
Propylene	5	(3·5)	*Trans* 2-butene	0·3	(0·20)
1-butene	7	(4·5)	*2-pentene	0·8	(1·0)
1-pentene	10	(13)	*2-hexene	1·2	(4)
1-hexene	14	(45)	*3-heptene	2·0	(20)
Oxygen compounds			**Miscellaneous**		
Ethyl vinyl ether	0·3	(0·5)	Cyclopentene	0·15	(0·3)
Butyl vinyl ether	0·3	(3)	Cyclohexene	2·0	(12)
1,2 dihydro-pyran	0·25	(2)	Cycloheptene	0·8	(10)
Anethole	0·25	(2400)	2-Me-2-butene	0·5	(0·45)
Crotyl alcohol	0·7	(70)	2,4,4 trimethyl 2-pentene	2·0	(25)

* Commercial mixtures of *cis* and *trans*.

Main series of olefins

The results of the bactericidal tests are given in Table 2. The EC/BG or MA/BG ratio after exposure to olefin alone was taken as the baseline throughout, varying from experiment to experiment between 1 and 4. The ratio in the spray solution was also found, by plating out a sample before the experiment, and it was found that never less than 50% of either MA or EC survived the process of spraying, collection on microthreads, exposure to olefin and rehydration in phosphate buffered sucrose/alginate solution. At lower relative humidities EC does not survive so well (see below), but on the whole it is clear that frames prepared in this way make a satisfactory test vehicle for the population being tested.

Effect of relative humidity

The main series of experiments was done at a high R.H. because most air disinfectants show maximum activity in this region (Nash, 1962). Some experiments were also done however with one olefin, cyclohexene, over a range of humidities. As expected, there was good survival in olefin vapour alone throughout the R.H. range for MA, but poor survival at the low end for EC, down to 20% of the spray solution ratio at R.H. 40%. As in the main series, however, survival after exposure to olefin vapour alone, whatever the R.H., was taken as the baseline, and the results are plotted in Fig. 1.

Table 2. *Survival of microorganisms on microthreads after exposure to ozone–olefin mixtures.*

(Olefin concentrations are as in Table 1. Initial ozone concentrations are in parts per hundred million (p.p.h.m.). Ten minute exposures at 19–22° C., relative humidity 76–79 %. Figures are percentage of the survival when exposed for the same time to olefin alone; the first figure is for *Micrococcus albus*, the second for *Escherichia coli*.)

	Initial ozone concentration, p.p.h.m.		
Olefin	0·4	1·1	3·3
Cycloheptene	10, 10	4, 6	—
Methyl cyclopentene	15, 10	7, 4	5, 3
Cyclopentene	25, 15	6, 6	3, 2
Dihydropyran	25, 35	2, 6	3, 3
Anethole	45, 55	4, 7	3, 2
Cyclohexene	45, 35	15, 7	2, 2
Crotyl alcohol	65, 55	15, 10	2, 5
2-hexene	65, 70	15, 20	5, 5
2-pentene	45, 80	10, 50	5, 10
Petrol, top	95, 90	30, 35	10, 10
1-pentene	80, 65	50, 20	10, 5
Trans 2-butene	70, 85	45, 30	10, 10
3-heptene	80, 90	25, 45	10, 35
Petrol, bottom	100, 70	50, 30	15, 20
Propylene	80, 70	55, 70	10 ,20
1-hexene	85, 75	50, 70	15, 20
1-butene	90, 90	90, 35	50, 35
cis-2 butene	*	75, 75	40, 20
2-methyl 2-butene	*	80, 75	20, 55
Vinyl butyl ether	*	100, 70	50, 45
Vinyl ethyl ether	*	*	25, 30
2,4,4 trimethyl pentene	*	*	*

* Not significantly different from 100.

DISCUSSION

The olefins listed in Table 2 are placed roughly in order of activity, averaging figures for the two organisms. A limitation of the microthread technique as used here is that about 2 % of the organisms are deposited on the frames, where they are to a large extent protected against toxic vapours. Survivals of 2 or 3 % may therefore indicate considerably greater kill on the microthreads themselves.

It can be seen first of all that there is perhaps a rather poor correlation between reactivity with ozone (Table 1; least p.p.m. denotes greatest reactivity) and bactericidal effect. The most significant correlation is probably that between structure and activity, in that all the ring olefins are at the top of the table with anethole, which is also a ring compound although the olefinic portion in this case is outside. This correlation fits in with what is known about the ozone–olefin reaction, together with the well-established principle that a good air disinfectant must have a very low vapour pressure (Nash, 1951).

Attack by ozone splits the double bond, one end becoming ketone or aldehyde

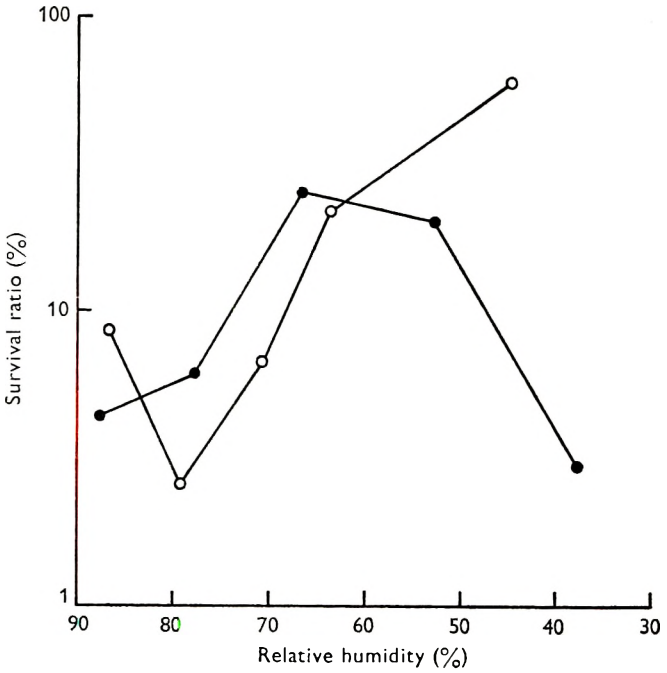

Fig. 1. Survival on microthreads exposed to mixtures of ozone (1·1 parts per hundred million) and cyclohexene (2 parts per million) mixtures for 10 min. at different relative humidities. ●, *Escherichia coli* strain 162; ○, *Micrococcus albus*, N.C.T.C. 7944. Both organisms sprayed in distilled water. Survivals are expressed as percentages of the survivals of the organisms exposed to cyclohexene vapour alone for the same length of time.

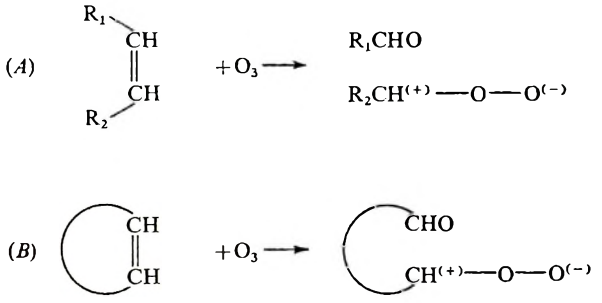

Fig. 2

and the other a peroxide 'zwitterion', this being probably the active fragment (Fig. 2). Comparing two olefins of the same molecular size, one a ring and the other an open chain, it is clear that the active fragment from the open chain olefin will be smaller and have a higher vapour pressure than the product from the ring olefin, where the molecule cannot be split into two separate portions.

Chemical constitution must not be neglected, but there is no point in drawing any further inferences at this stage without knowing the exact course of the ozone reaction in each case. For instance, it is difficult to see why the two butenes should differ so much in activity, when the products of ozonation should be the same.

It is possible that recombination of the fragments to form true ozonides, which occurs at high concentration, also occurs at low concentration and is critically dependent on constitution. There is scope here for a more detailed investigation of selected olefins.

There is one further aspect of the results which deserves some discussion, that is the concentration at which the ozone–olefin reaction products are bactericidal in comparison with the concentrations at which known air disinfectants are bactericidal. Taking resorcinol as an example, the amount required for a good kill is about 100 times the amount of ozone, if a suitable olefin is present. There is a simple physical explanation of this, going back to the known behaviour of triethylene glycol (Nash, 1951). When this substance is vaporized from a hot plate, for air disinfection tests, it condenses again to a cloud of droplets, because the vapour pressure is exceptionally low. Its (rather poor) bactericidal effect is mainly due to vapour slowly distilling from these droplets onto the bacteria-carrying particles. It is easy to imagine that with compounds of still lower vapour pressure bactericidal action will get less and less, from the same quantity of material, because the rate of production of vapour from the condensed aerosol will get less and less and finally be so small that aerial disinfectant activity will be reduced to zero. A substance whose vapour pressure is a hundred times less than that of resorcinol may well be active at a 100-fold less concentration of vapour, but this concentration would never be approached in practice because of aerosol formation immediately after vaporization. The ozone–olefin reaction products, on the other hand, are produced initially as single molecules in the very dilute gas phase, with the maximum chance of condensation on to bacteria-carrying particles, or any other surface in the neighbourhood.

There is no need, therefore, to postulate exceptional bactericidal activity on the part of these products, but merely exceptionally favourable physical circumstances.

REFERENCES

Anderson, J. D. (1966). Biochemical studies of lethal processes in aerosols of *Escherichia coli*. *Journal of General Microbiology* **45**, 303.

Brewer, A. W. & Milford, J. R. (1960). The Oxford–Kew ozone sonde. *Proceedings of the Royal Society, London*, Series A **256**, 470.

Bufalini, J. J. & Altshuller, A. P. (1965). Kinetics of vaporphase hydrocarbon–ozone reactions. *Canadian Journal of Chemistry* **43**, 2243.

Druett, H. A. & May, K. R. (1968). Unstable germicidal pollutant in rural air. *Nature, London* **220**, 395.

Druett, H. A. & Packman, L. P. (1968). Sensitive microbiological detector for air pollution. *Nature, London* **218**, 699.

Leighton, P. A. (1961). *Photochemistry of Air Pollution*. London: Academic Press.

May, K. R. & Druett, H. A. (1968). A microthread technique for studying the viability of microbes in a simulated airborne state. *Journal of General Microbiology* **51**, 353.

Nash, T. (1951). Physical aspects of air disinfection. *Journal of Hygiene* **49**, 382.

Nash, T. (1962). The bactericidal properties of compounds which protect living cells against freezing damage. *Journal of Hygiene* **60**, 353.

Nash, T. (1967). Colorimetric determination of ozone by diacetyl-dihydro-lutidine. *Atmospheric Environment*, **1**, 679.

J. Hyg., Camb. (1970), **68**, 253
Printed in Great Britain

Studies on the 1967–8 foot-and-mouth disease epidemic

The relation of weather to the spread of disease

By M. E. HUGH-JONES
Central Veterinary Laboratory, M.A.F.F., New Haw, Surrey
and P. B. WRIGHT
Meteorological Office, Bracknell, Berks.

(*Received* 16 *January* 1970)

SUMMARY

An analysis of the 1967–8 foot-and-mouth disease epidemic with reference to the initial spread, the origin of outbreaks more than 60 km. from the main epidemic area, the series of outbreaks near Worcester, a specific case history and the daily rate of spread of the epidemic, strongly suggests that the weather played a major part in the spread of disease. The two main factors involved in this type of spread are wind and precipitation. It is noted that after the epidemic had been checked, following anticyclonic weather, the association between the weather and the spread of disease was less apparent.

INTRODUCTION

Many possible means of spread of the virus of foot-and-mouth disease (FMD) are known or have been suggested. It is believed that the virus can be carried by any of the following agents: infected or incubating animals, bones or meat or milk from such animals, by fomites such as birds, insects, rats, hay, vehicles or people and by the wind.

During the 1967–8 epidemic in England and Wales, every outbreak was carefully investigated by the Government Veterinary Field Service (V.F.S.) to try to identify the most likely cause; however, the number of outbreaks for which a definite origin was eventually determined was very small—of the order of 5%. The source of infection for some 2200 outbreaks remained unaccounted for.

The possibility that foot-and-mouth disease virus may be disseminated by the wind was first put forward by Hardy & Milne (1938), who suggested that it was carried on particles and insects, and by McClean (1938), who suggested that it might be absorbed on aeroplankton yeasts. Experimental airborne infection over 10 m. has been demonstrated by Fogedby, Malmquist, Osteen & Johnson (1960). Hyslop (1965*b*) showed the presence of aerosols of FMD virus in air, coarse-filtered to remove dust, from loose boxes containing infected cattle; his work (1965*a*) also demonstrated that release of virus into the air occurred before the appearance of clinical symptoms, as well as during and after the appearance and healing of vesicles.

Some evidence has been advanced in favour of the possibility of airborne spread over distances up to 150 km. Fogedby *et al.* (1960) described how the disease appeared in Scandinavia after being present in northern Germany, and how it appeared to 'hop' from island to island in the Danish archipelago. Hurst (1968) has shown that many of the primary outbreaks which have occurred near the east and south coasts of England since 1937 can be attributed to wind-borne spread from sources on the Continent, although the first outbreak in the 1967–8 epidemic was not windborne. Most recently Smith & Hugh-Jones (1969) demonstrated that the wind has been one of the prime factors in the spread of FMD during previous epidemics in this country, and that most outbreaks due to wind spread were associated with rain. The present paper will demonstrate that during the 1967–8 epidemic the weather also played a very large part in the spread.

METHODS

Disease data

Some of the disease data were gathered during the period 5 Nov. 1967 to 14 Jan. 1968 by one of us (M.E.H.-J.), who interviewed farmers and veterinary officers in selected areas and maintained an index of map references of affected stock and premises. In July–August 1968 further information was abstracted from the V.F.S. files of all outbreaks; this included date of confirmation of FMD, the total number and species of animals reported diseased, number and species of animals in contact with affected stock, date and time of slaughter of the latter group, the number and species of stock affected each day until slaughter; whether stock were housed or grazing, type of housing and how long they had been in this accommodation before official confirmation of foot-and-mouth disease. Whilst it is agreed that this hearsay information is not as accurate as that obtained by individual interviews, the size of the epidemic made the latter impossible in every case. The information in the files was also used to estimate the most probable date of first clinical disease for each affected group, accepting that even in controlled circumstances it is an inspired guess. When an adequate herd history was available, the date was taken to be the earliest date on which any animal, later found to have lesions of foot-and-mouth disease, had been noted by the owner or his staff, his veterinary surgeon or visiting veterinary officer to be 'off colour', lame or suddenly producing less milk. Otherwise the date was estimated by the extent and condition of the recorded lesions and clinical record of the affected stock. It was assumed that, for cattle and pigs, symptoms occurred at the following intervals from onset of disease: temperature of 105°–106° F. (at onset), unruptured vesicles (4–6 hr.), recently ruptured vesicles (6–8 hr.), ruptured vesicles with ragged edges and strips of loose epithelium (12–18 hr.), separation of horn from coronary band and necrosis (24–36 hr.), granulation tissue (36 hr. or more). For sheep, however, the estimation of the age of clinical disease in a flock using this method is extremely unreliable. When a farm had two or more outbreaks, widely separated in time or space, they were regarded as separate outbreaks. The period during which an outbreak was active as a source of infection was taken to be from the date of initial disease to

the date of slaughter; however, this may not be correct, not only because of doubts about the former date, but also because it is believed that animals may produce substantial quantities of virus before clinical disease appears (Burrows, 1968).

Meteorological data

Hourly weather records from the Meteorological Office at R.A.F. Shawbury, situated 8 miles north of Shrewsbury, were considered to be adequately representative of the general weather conditions over the epidemic area as a whole and were used in most of the analyses. Data from the Pershore meteorological stations were used in the analyses of the small epidemic near Worcester.

Detailed analysis

Detailed studies were made of the spread during the initial and final stages of the epidemic, and in Worcestershire. In these studies an attempt was made to determine the most likely source of each individual outbreak, taking into account wind directions, rain, proximity to the supposed source and variations in the incubation period. Various indications (M. E. Hugh-Jones, unpublished work) led us to believe that during this epidemic the incubation period lay between 4 and 14 days, with a mode at about 8 days. The minimum interfarm disease interval seemed to be 3 days; because of the high viability of the virus strain in this epidemic, a maximum interval was difficult to estimate owing to the delay by susceptible animals in finding an infective dose of virus.

This type of analysis was not practicable for most of the epidemic because of the multiplicity of possible sources of each outbreak.

Statistical analysis

This analysis was an attempt to relate the amount of spread at different stages of the epidemic to meteorological and other possible influences. It was necessary to calculate for each day an estimate of the 'rate of spread'; this was defined as:

$$\frac{\text{Apparent number of outbreaks resulting from spread on day D}}{\text{Source strength on day D}}.$$

The denominator was calculated by the same method as was used by Smith & Hugh-Jones (1969), using outbreaks in cattle and pigs. Outbreaks in flocks of sheep (which formed less than 5% of the total number of outbreaks) were omitted because of the frequent doubts about their initial disease dates, and also because infected flocks, for reasons little understood, do not appear frequently to infect stock grazing adjoining fields. The numerator depends on the length of the incubation period, and we took it to be the mean number of outbreaks with initial disease on days D+7, D+8, D+9, again omitting sheep outbreaks. For the period from 15 Jan. onwards, because of the small number of outbreaks, 5-day mean values were calculated. For the first 11 days of the epidemic it was possible to estimate the rate of spread a little more accurately. The resulting estimates are included in Fig. 6.

THE INITIAL SPREAD

The first known outbreak and that with the longest history of clinical disease was Bryn Farm at Nant Mawr, 5 miles to the south of Oswestry in Shropshire. Briefly, one sow was lame on 21 Oct. 1967, a second on 23 Oct., a third sow and eleven store pigs were ailing on 25 Oct. and by slaughter the next day a total of 28 pigs were found to be diseased. The farms next affected in the area were two neighbours within ½ km. and 2 km. with 12 and 14 affected cattle on 27 Oct. It is possible that there were other unrecognized affected stock in the area before or concurrent with the Bryn Farm outbreak, but this can be ignored as it does not affect the following argument.

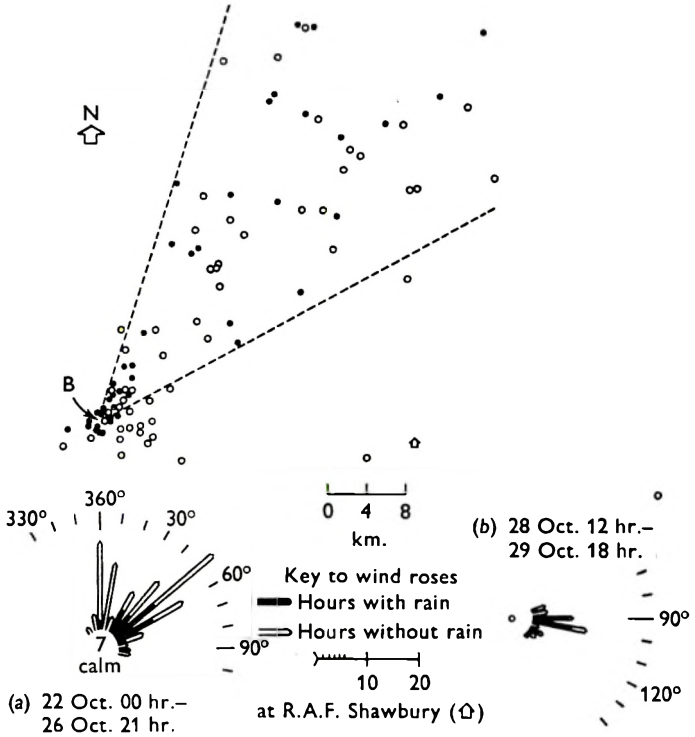

Fig. 1. Distribution of FMD outbreaks during the periods 21 Oct.–1 Nov. 1967 (dots), and 2–5 Nov. 1967 (open circles).

Figure 1 shows (dots) the positions of all the outbreaks in the country up to 1 Nov., with the exception of one at Carnforth, Lancashire. It can be seen that 31 outbreaks lay within a limited sector (between discontinuous lines), and 11 outbreaks were outside this sector but less than 2 km. from Bryn Farm, the vertex. Only two outbreaks were outside the sector and more than 2 km. from Bryn Farm; one of these was 3 km. to the WSW, the other was the Carnforth outbreak 150 km. away on bearing 010°.

Figure 1*a* shows that during the period 22 Oct. 00 hr. to 26 Oct. 21 hr. the wind blew predominantly towards bearings 360°–070°. This range of directions contains the sector in which the outbreaks occurred. Most of the outbreaks outside the sector were so near to the source that temporary or local variations of wind, or

winds on 21 Oct. which included calm spells, could have been associated with them. It rained frequently during the period 22–26 Oct. It is not possible to associate any of the outbreaks with any specific type of weather, although there is some suggestion that spread was confined to the directions in which the rainy winds blew. However this association may be partly due to other differences such as variations in the strength of the source; winds blowing towards the north did not occur after 13.00 hr. on 25 Oct., and since the number of known clinically affected animals at the source gradually increased, more spread could have been expected towards the end of the period than at the beginning.

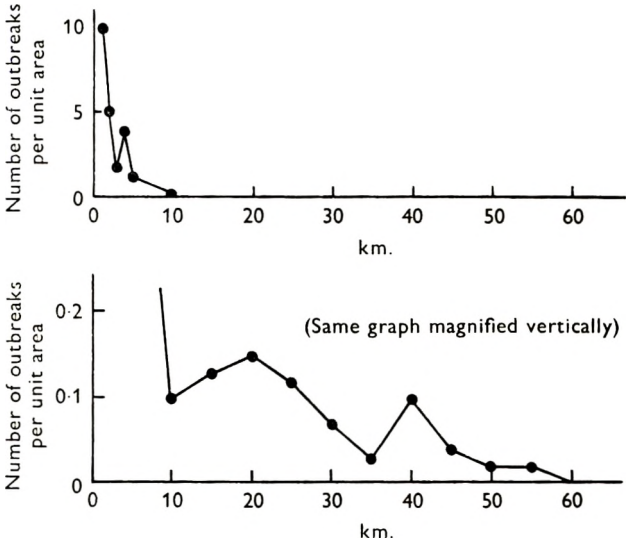

Fig. 2. Number of outbreaks, 21 Oct.–1 Nov., per unit area, plotted against distance from the first outbreak. Only farms which lay between the dotted lines of Fig. 1 are included.

Figure 2 shows the number of farms per unit area affected at different distances downwind in the sector delineated in Fig. 1. The distribution shows a decrease approximately with the square of the distance from the vertex, conforming to the concentration pattern which would result from windborne spread of material from a point source.

The mean bearing of the 19 outbreaks within the sector and more than 10 km. from Bryn Farm was 039°. The mean surface wind direction (21 Oct. 23 hr. to 26 Oct. 22 hr. based on hourly values) was 034°, and the mean 2000 ft. wind direction (22 Oct. 00 hr. to 26 Oct. 21 hr. based on 3-hourly values) was 068°. Thus the direction of spread was closer to the surface wind than to the 2000 ft. wind. Pasquill (1961) gives a formula for calculating the position of the axis of a plume of windborne material at distances of 10–100 km. downwind of a source:

Bearing of axis =

$$\tfrac{1}{2}(\text{mean surface wind bearing} + \text{mean 2000 ft. wind bearing}) - 10°.$$

In the present example the formula yields the value 041°, close to the mean

bearing (039°) of the outbreaks. The height at which transport took place was somewhat above the surface, but not nearly as high as 2000 ft.

The outbreaks which first showed disease during the period 2–5 Nov. are also shown in Fig. 1 (open circles). Most of these were in the same sector as the earlier outbreaks, and while some of them may have had the same source, others may have been the result of spread from the earlier outbreaks; so individual analysis is of little value. However, of particular interest are the 12 which occurred more than 2 km. from Bryn Farm in a sector to the east of Nant Mawr; 10 were in a cluster within 8 km., the other two were respectively 27 km. and 57 km. away. No previous outbreaks had occurred in this region, and this suggests that these 12 must have become infected at a later date than the earlier outbreaks. If the minimum possible incubation period is assumed to be 4 days, it follows that infection would have occurred in the period 27–29 Oct. During this period there were within 2 km. of Bryn Farm three farms with diseased cattle and one with diseased sheep. From 12.00 hr. on 28 to 18.00 hr. on 29 Oct. the wind blew with rain mainly towards bearings 090°–100° (*b*, Fig. 1) with short spells on either side of this range. The 12 outbreaks were centred on these directions, and in particular the two outlying outbreaks both lay on a bearing of 099° from one of the farms affected on 29 Oct. and at distances comparable to those reached by the previous wave of infection.

It may therefore be concluded that most of the initial spread in the 1967–8 epidemic can be attributed to wind.

LONG DISTANCE SPREAD

There were thirteen outbreaks (approximately 0·5 % of the total) which were at least 60 km. from any possible source and for which no fomite was identified as being responsible for the spread (see Table 1). There may have been some further

Table 1. *Outbreaks which were probably at least* 60 *km. from their source*

Area	Approximate distance from main infected area* (km.)	Date of initial disease	If wind-spread, probable time when spread occurred
Carnforth	150	29. x.	22. x.–25. x.
Darley Dale	70	6. xi.	22. x.–1. xi.
Blackburn	60	9. xi.	1. xi.
Leicester	70	18. xi.	9. xi.–14. xi.
N.W. Gloucestershire	90	21. xi.	15. xi.–16. xi.
Usk	110	23. xi.	15. xi.–16. xi.
North Hykeham	110	7. xii.	27. xi.–30. xi.
Castle Bytham	120	8. xii.	27. xi.–30. xi.
Ledbury	80	16. xii.	6. xii.–12. xii.
N. Herefordshire	60	22. xii.	16. xii.–17. xii.
Darley Dale	60	29. xii.	21. xii.–24. xii.
Coventry	60	9. i.	30. xii.–3. i.
Market Harborough	80	13. i.	3. i.–6. i.

* Cheshire–North Shropshire.

outbreaks, on the fringe of the main infected area, which resulted from spread over distances of 60 km. or more, but these are difficult to identify. These outbreaks may have been caused by wind or some other mechanism, while some may have been primaries resulting from an untraced or hypothetical source. Mead's work (1968) suggests that spread by birds must be considered unlikely.

In each case there was a wind blowing with rain from the main infected area at a suitable time. The distribution of distances (for what it is worth, being based on such a small data sample) conforms well with a wind hypothesis.

A description of the circumstances of two of these outbreaks at North Hykeham and Castle Bytham is of interest. Diseases first occurred in Lincolnshire on 7 Dec. at North Hykeham, just south of Lincoln, in 37 heifers and a bull grazing a 10-acre field without cover or shelter. By slaughter at 11 hr. on 9 Dec. every animal in the herd was affected. It is possible that one animal may have been infected a few days previously and infected the others, but clinical disease appeared in all animals within only 35 hr., which is extraordinarily fast for spread within a grazing herd, the owner had been regularly inspecting them thrice each day, and a thorough examination of the slaughtered animals revealed none with healing lesions. It would therefore seem probable that virus was deposited on the field or feeding trough and infected all the animals nearly simultaneously. A thorough tracing by veterinary officers revealed no contacts with the main epidemic nor any other suspected sources of infection. The nearest outbreak at this time was 70 km. to the west and the main area 110 km.; however, winds blew from the west throughout the period 27–30 Nov., with intermittent rain each day.

On 8 Dec. one housed cow out of 26 was affected at Castle Bytham, 44 km. south of North Hykeham; there had been no previous outbreaks in the area.

It is not unlikely that these two outbreaks were extreme instances of airborne spread and intermittent rain deposition. We have noted that the furthest outbreaks in such circumstances often occur in a single line or front at right angles to the probable direction of spread.

The Worcester sub-epidemic

Between 14 Nov. and 6 Dec. 38 outbreaks occurred in a small area of Worcestershire just east of Worcester. Infection was introduced to three farms in this area in skim milk and there were no outbreaks in adjoining areas which can reasonably be supposed to have been the result of long distance spread from the main epidemic; the area can therefore be treated in isolation.

On 10 and 13 Nov. 500 gallons of skim milk were delivered to farm 1, 800 gallons to farm 2, and 500 gallons to farm 3. Other outbreaks elsewhere at the same time also traced to this skim milk indicate that the 10 Nov. delivery was most likely to have been infectious. At farm 1 the skim milk was fed to, among others, two sows and their 19 8-week-old offspring in a pen in an open-fronted shed. One sow was noticed to be 'poorly' on 14 Nov. At slaughter on 16 Nov. both sows and three young were clinically affected, and the next day 9 of the remaining 15 were affected. As a result of this outbreak, regular inspections were made of the other two farms which had received milk from the same consignment. At farm 2

4/50 store pigs (10–12 weeks old) in an open-fronted pen and fed on skim milk, were first noted to be ill by the owner at mid-day 17 Nov., with a total of 14 clinically affected at 17 hr. when slaughtered. The first pig noted to be ill at farm 3 was a newly weaned pig in a litter of eleven 10-week-old stores on 18 Nov. By slaughter the next day two more were affected. The store pigs in an adjoining fattening house fed the same skim milk were apparently healthy at slaughter.

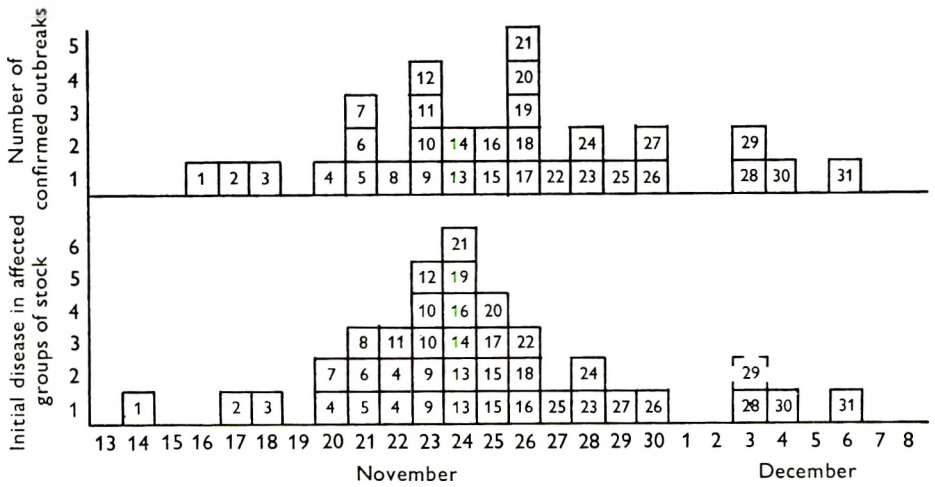

Fig. 3. Dates of the confirmation and the estimated first appearance of disease in herds and flocks in the Worcester sub-epidemic. The internal spread of disease on two farms, 19 and 21, has been disregarded.

Thorough tracing of bulk tankers and milk lorries by veterinary officers could not implicate milk in any of the subsequent spread in this area. Nor could any mechanical, human or animal vehicle be blamed, except on one farm (19), where infection was probably spread internally by a shepherd tending two flocks, and another (21), where outlying stock were brought into contact with the housed home stock. On some farms clinical disease appeared in different parts of the farm coincidently or had developed by the time of slaughter. If these outbreaks are re-plotted by date of initial disease in different affected herds or flocks instead of confirmation date of disease on the farm, it can be seen that most of these outbreaks occurred within 13 days of the last pig killed on farm 1 on 17 Nov. (Fig. 3). The majority of outbreaks formed a cluster about farm 1 (Fig. 4). A careful tracing, counting and plotting of all the herds and flocks in the area showed that there were more than adequate numbers of susceptible stock on nearby and more distant farms for the epidemic to have spread further and especially from farms 2 and 3.

During the period 14–17 Nov., when farm 1 was infected, the winds varied in direction and there was rain at times while the wind was blowing towards the east (see Appendix for weather summary). The subsequent outbreaks about farm 1 were distributed in accordance with the weather during this period. In contrast, farm 2 was probably an active source on 17 Nov. and farm 3 on 18–19 Nov. for farms 18, 25 and possibly 29; this was a time of dry weather with light easterly winds, and

a relative absence of new outbreaks. During 20–24 Nov., when there was the maximum number of outbreaks, adequate numbers of animals available in spite of the slaughter of 'dangerous contacts', and dry anticyclonic weather with calm or light winds, little if any spread occurred; if and when spread did occur at this time (it is difficult to decide whether the individual outbreaks 23, 24 and 26, represent new infections or normal but long incubation periods), it was in the nature of infilling and not centrifugal dispersal. On the farms where FMD was diagnosed, virtually all groups of stock were affected by the time of slaughter and

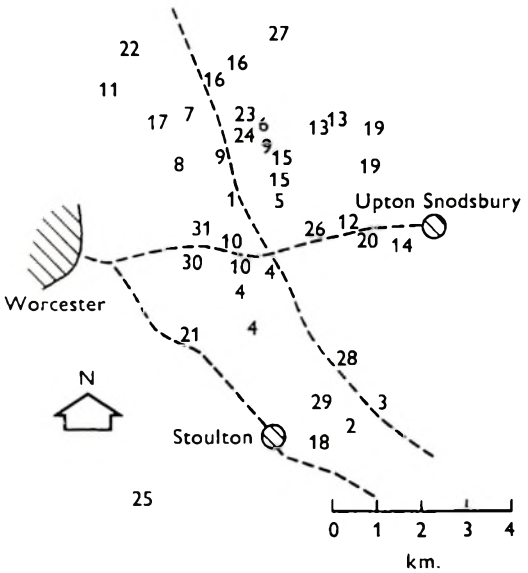

Fig. 4. Outbreaks in the Worcester area. The outbreak number for a farm is repeated for as many separate groups of stock as were clinically affected on the farm and indicates their positions.

it is only at the margins that unaffected groups were common, allowing for slaughter limiting the expression of disease. The commonest groups which apparently escaped disease were sheep flocks: it is notoriously difficult to diagnose FMD in sheep flocks. With the reappearance of wet weather, further outbreaks occurred. During 3 hr. of the early morning of 27 Nov., 1 mm. of rain fell with the wind blowing due north. Disease appeared in cattle at farms 30 and 31, 3 km. north of 20 affected cattle at farm 21, and at farm 28 adjacent to farms 18 and 29. So many sheep were affected at farm 29 that it was impossible to ascertain the initial date of disease in the flock. The earlier period of light rain on 25 Nov. fell with the wind blowing towards areas without stock and when these hypothetical 'source' farms had fewer diseased stock.

The study of this sub-epidemic was carried out during the epidemic and agrees with Henderson's results (1969). However we are not as confident as he as to the source of infection of some of the later outbreaks.

Although foot-and-mouth disease is extremely infectious, this was a very localized epidemic which affected virtually all the stock in the original dispersal

and stopped in spite of the nearby availability of susceptible stock. This check occurred coincidentally with the anticyclone.

A specific history

This case history is presented to demonstrate a specific example of an aerial, and possibly aerosol, spread of infection between two herds.

Farmer A and Farmer B share what was once one set of farm buildings (Fig. 5). Both farmers kept dairy cattle in these buildings, but as far as could be ascertained had taken reasonable precautions to isolate themselves and their stock from each other. There was no common airspace, such as a loft or eave spaces between the two herds, and the brick wall dividing the two cowsheds was about ten inches

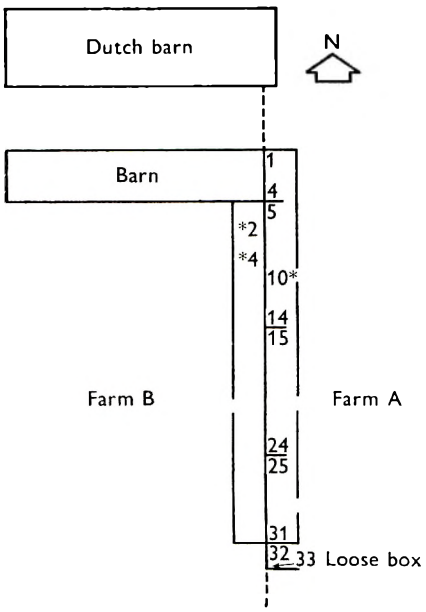

Fig. 5. Farms A and B showing standings (numbered). Asterisks indicate first animals affected on each farm.

thick with slots every 15 ft. and 8 ft. from the floor. These slots, 6 in. × 12 in. × 10 in., were filled with rubble and bricks except for the slot next to Farmer A's standing 10, which was filled with hay only.

On 16 Nov. at 07 hr. Farmer A noticed that a 6-year-old in-milk Friesian cow in standing 10 was dull, shivering, hunched up, salivating slightly with a temperature of 102·2° F. but eating normally. By 10 hr. her temperature had risen to 105·5° F. and he telephoned the nearest Foot-and-Mouth Disease Centre. A veterinary officer visited the farm at 11 hr. and found the cow to be 'off-colour', depressed, shivering, lifting her feet, with a temperature of 106° F. but without vesicles in the mouth or on the udder. Not convinced, but suspecting FMD, he served on the farm a formal restricting order and re-examined the cow at 17 hr., when the cow had a temperature of 105° F. but was otherwise no different and picking at her food. He saw her again the next morning at 10 hr., when she had

a temperature at 103° F., was much 'brighter', feeding, and without any vesicles in the mouth or on the feet and udder. Because of her habit of over-eating the officer diagnosed the condition as laminitis and removed the restriction. Later that morning, 17 Nov., Farmer A moved her into standing 33 in the loose box and put a cow from the loose box in her standing.

At about 22 hr. on 18 Nov. Farmer A noticed that five cows about standing 10 had become uneasy, and the next morning his veterinary surgeon diagnosed foot-and-mouth disease. At mid-day on 19 Nov. the cows in the following standings had unruptured or recently ruptured vesicles in the foot or mouth, cows 1, 4, 5, 9 to 14, 17, 19, 24, 27, 33, and cows in standings 20, 21 and 30 had temperatures over 104·5° F. The cow in standing 33 had healing lesions in her mouth and extensive separation at the coronary bands of her feet.

Farmer B's 27 milking cows were slaughtered as dangerous contacts on 20 Nov. at 15 hr. The cows in standing 2 and 4 opposite to Farmer A's standings 6 and 10, were found to have high temperatures and vesicles. The cow in standing 4 was opposite the hay-filled slot.

The hay-filled slot above standing 10 would have acted as a coarse filter and allowed through an aerosol, but probably not dust, drawn through as a result of any minor pressure differences between the two air spaces. It is highly possible that Farmer B's cows were infected as a result of aerosol transmission through this slot.

Epidemic pattern

If we assume for the moment that all areas respond with equal numbers of outbreaks to equal amounts of virus deposited in them, we may use the daily 'rates of spread' as defined under Methods, to study the behaviour of the epidemic. The spread from Bryn Farm (most of which took place on 25 and 26 Oct.) resulted in 0·7 secondaries per unit source, much higher than at any later period of the epidemic. The spread then settled down to a general figure near 0·3 secondaries per unit source until mid-November. There was then a rapid drop in the rate of spread to 0·1 secondaries per unit source, as the epidemic passed its 'peak' (the maximum daily totals of confirmed cases were during 21–26 Nov., the spread which caused them being therefore about 15 Nov.). After a small rise in the rate of speed to about 0·17 in early December, it gradually decreased to below 0·1 by the end of the month. Throughout November and December there were large fluctuations with no clear general figure, but the details for these months must be treated with caution because of the small samples involved.

Comparison of the rate of spread with wind speed shows evidence of association (Fig. 6). In particular, the rate of spread was exceptionally high on 25–26 Oct., and on both these days the wind speed also was higher than on most days of the epidemic and was also constant in direction. Other marked similarities between the two graphs are the peaks on 5–6 Nov., the troughs about 9 Nov. and the peaks about 15 January. There were, however, many variations in the rate of spread which were not associated with similar variations in wind speed. It is of interest to note that from late October to mid-November maxima of wind speed occurred

about every sixth day. This interval is similar to that between infection and viraemia, if we assume a modal incubation period for clinical disease of 7–9 days.

The most important similarities between the graphs of rate of spread and precipitation duration were the coincidence of the steep drop in the rate of spread in mid-November with the start of the first dry spell of the epidemic, and also the

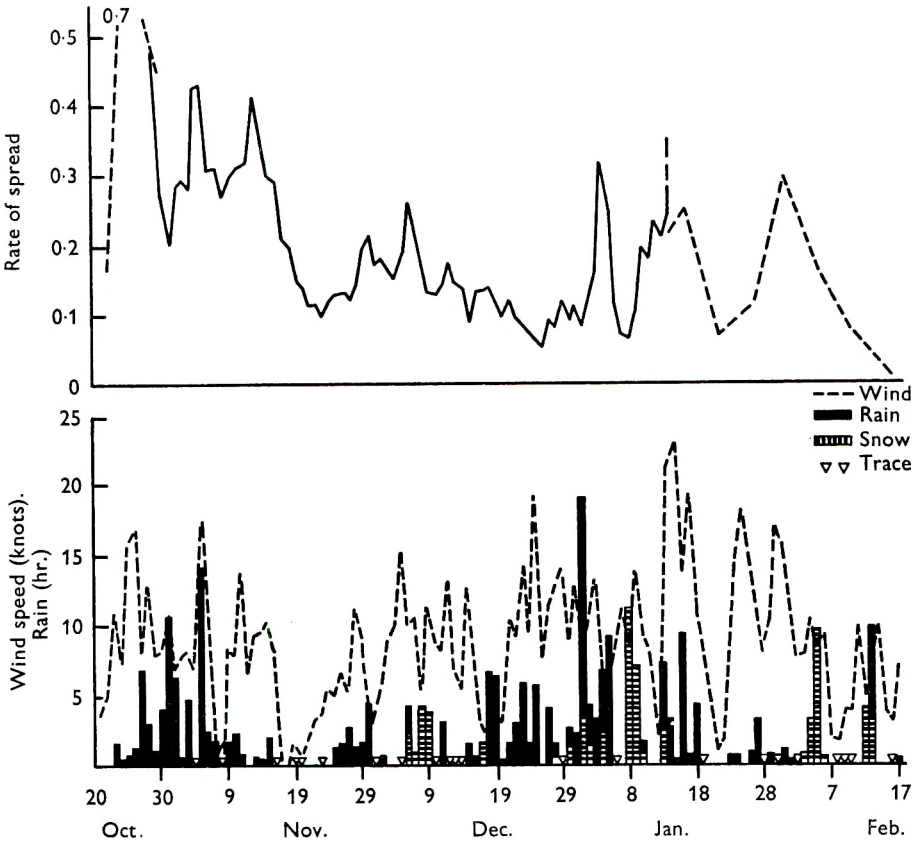

Fig. 6. The daily rates of spread of disease, mean wind speed (knots) and hours of precipitation during the period 21 Oct. 1967–17 Feb. 1968.

coincidence of the subsequent rise in the rate of spread with the return of rain towards the end of November. After this dry spell, the three highest peaks in the rate of spread were associated with three periods of snow—6–9 Dec., 8–10 Jan., and 3–5 Feb.; snow is known to be a highly efficient interceptor of airborne particles. However, while the snow in early December coincided with the peak, the other snow periods appear to have been somewhat later than the corresponding peaks. The association with hours of precipitation was not always close; for example, while the wet spell in early November was accompanied by a high rate of spread, a nearly equally wet spell (with similar average wind speeds) during Christmas week was accompanied by the lowest rate of spread of the epidemic. During the period 30 Oct.–20 Nov., a time of greatest spread, there was an average of 2·3 hr. rain per day; 21 Nov.–31 Dec. during least spread, 1·6 hr. rain per day;

1 Jan.–13 Feb. during increasing spread there were 2·6 hr. rain per day. Precipitation duration and amount were highly correlated, and so a comparison with amount would yield similar results.

There is a closer association between the daily rates of spread and the weather variables (01–24 hr.) during the period 30 Oct.–20 Nov. than at any other time of the epidemic. The correlation coefficients are:

	Factors	1	2	3	4	
1	Rate of spread	1	—	—	—	
2	Rain duration (hr.)	0·20	1	—	—	
3	Mean wind speed (kts)	0·53	0·57	1	—	
4	Mean wind speed during rain (kts)	0·38	0·58	0·85	1	
5	24-hour variation of wind direction (°)	0·00	0·24	0·16	0·16	1

(5 % Significance level, 0·40)

This table demonstrates two points, that the weather variables are inter-related, and that the mean daily speed more than the wind blowing only during periods of rain is most closely associated quantitatively with the daily rate of spread. This is not incompatible with the previous results associating the spread of disease with rain-bearing winds because the weather factors are closely inter-related and because this quantitative analysis is non-spatial. The correlation coefficients for the rest of the epidemic were insignificant.

There was a lower rate of spread after the peak than before. We have seen this diminished response to the weather in all other foot-and-mouth disease epidemics in the U.K. that we have studied.

Effect of housing stock

It was usual practice to advise only farmers contiguous to outbreaks to house as many stock as they could and to withdraw the remainder into the middle of the farm; presumably it was thought that most spread occurred over only short distances, and this procedure would therefore protect the animals. However, there is no evidence to suggest that housed animals were any less at risk (Wright, 1969; Henderson, 1969; M. E. Hugh-Jones, unpublished results). During the 1967–8 epidemic all farmers were advised to house their stock; this advice together with the normal seasonal trend towards housing of stock meant that the proportion of animals grazing decreased rapidly until about 20 Nov., after which date it remained fairly constant (Fig. 7). Housing of stock will greatly decrease the air-flow over them.

Effect of slaughter

As all epidemics appear to spread up to the peak in spite of the slaughtering of diseased stock, and in a more limited manner afterwards, slaughter cannot be regarded as an absolutely effective control. If it does have an effect after the peak, which is unclear before, the nature of this control must change. Until 26 Nov. more herds of pigs and cattle, in which disease had been diagnosed, were being slaughtered the day after confirmation than on the confirmation day, taking the

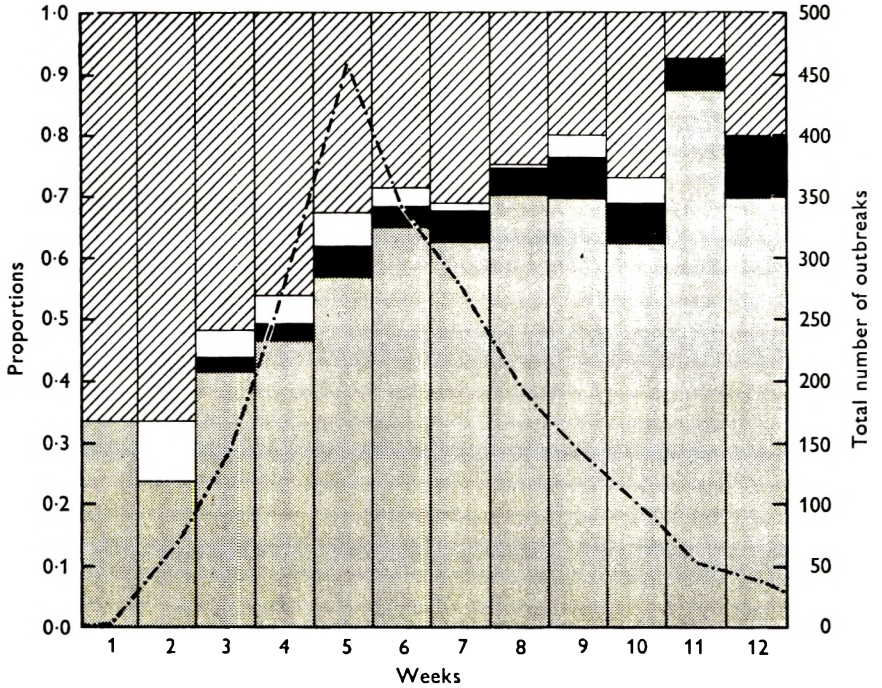

Fig. 7. Diagram showing proportions of infected herds of cattle housed (dotted), yarded (black) and grazing (diagonal lines) during each week of the epidemic; unrecorded or complex patterns of husbandry are blank. The graph of the number of outbreaks each week is shown for comparison.

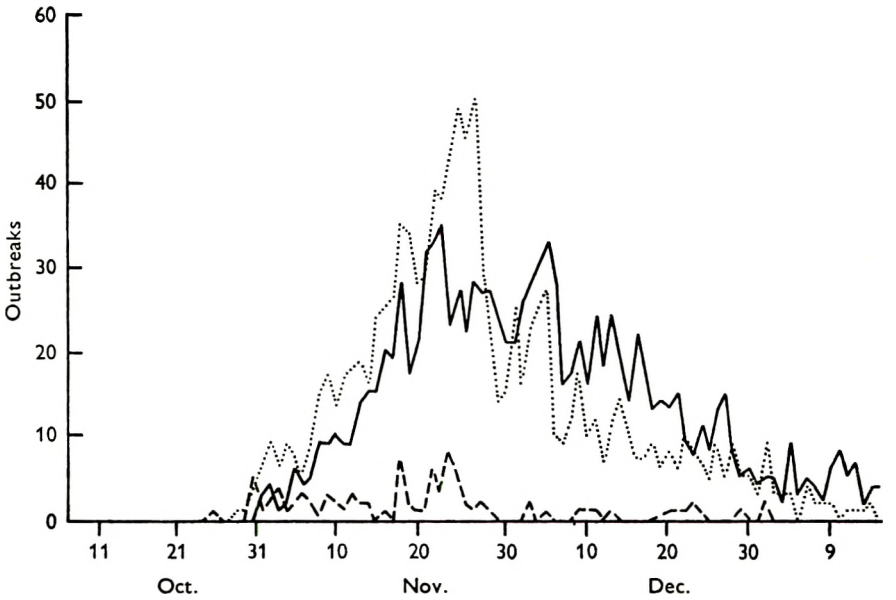

Fig. 8. Numbers of herds of cattle and pigs with confirmed FMD divided into three sets for each day: those which were slaughtered on the same day as confirmation, those slaughtered the next day, and those slaughtered more than one day later. ——, Same day; – – –, > one day; · · · ·, next day.

slaughter date to be the day on which animals in contact with the affected stock were killed. After this date the position was reversed (Fig. 8). During a 20-day period following 26 Nov. the average was 26·6 outbreaks in which the animals were killed on the same day as confirmation. The anticyclone in mid-November was associated with a reduction in the number of outbreaks to below 52 outbreaks per day; under these conditions more than 50 % of outbreaks were slaughtered on the same day as confirmation. This is in contrast to the early part of the epidemic when only a minority were being slaughtered on the day diagnosis was made.

DISCUSSION

The evidence from this epidemic clearly suggests that windborne spread does occur, and it is now necessary to put forward a hypothesis to explain the mechanism. This rests on several assumptions, which appear reasonable but await experimental verification. The virus must first get into the air in sufficient quantities to survive extensive dilution; it must be transported in the air; it must be brought out of suspension; it must remain sufficiently viable and concentrated during these processes for a susceptible animal to find it and be able to demonstrate disease symptoms.

Emission from an affected animal can occur by breathing, sneezing or salivation from entire or ruptured vesicles, or by splash from falling infective urine, milk or faeces. The greatest emission usually occurs just before lesions appear but it can occur several days before (Burrows, 1968). Some animals, especially sheep, will show no obvious signs of disease, and therefore can act as a 'silent' source of infection. The relative rates of emission into air by different species is not known but appears to be in the following descending order: pigs, cattle, sheep (R. Sellers, personal communication). Sheep appear to be poor emitters because stock near to a sheep outbreak are not often diseased, but this may be only an apparent effect due to the fact that adjacent groups are usually also sheep, which although becoming infected may miss being diagnosed.

The virus when emitted is probably contained in droplet nuclei of various sizes. If spread is to occur farther than to adjacent animals the virus must remain airborne for sufficiently long to travel the required distance; this is possible if it is attached to or incorporated in particles of suitable size. Particles of diameter greater than about 20 μ will be deposited by gravity within a few hundred metres of the source, although if the wind is strong they will be carried farther before being deposited. Smaller particles, on the other hand, will show little tendency to fall, and although there will be some deposition due to turbulent transfer, a large number could be carried very great distances. An increased wind speed would be expected to increase the number of the smaller particles by assisting in the dehydration and breaking-up of the larger droplets; this, together with the greater distance of travel of the larger drops, would account for the observation that high wind speeds were associated with increased spread. It would follow that the housing of infected animals would reduce the dissemination of virus as a result of decreasing the speed of air movement around the animals.

During the course of the spread the infective material will diffuse horizontally and vertically; formulae for calculating the distribution of particles of various sizes downwind of a source have been published by Pasquill (1961). The amount of diffusion is proportional to the time of travel, rather than the distance, hence if the wind is strong and constant in direction there will be a greater concentration of particles at any given distance, especially along the axis of the plume. The viability of virus aerosols in different conditions of pH, air pollution and ultra-violet radiation is not known, although they would all be expected to have an effect. The ability to produce recrudescences of disease on restocked premises up to 4 months after previous infections suggests that the virus is effectively stable in field conditions and that loss of virus occurs more through erosion than through reduced viability.

Deposition in wet and dry weather of particles of a suitable diameter has been described by Chamberlain (1955). In dry weather the amount of deposition would be expected to be comparatively small and localized. This is supported by the evidence from the 1967–8 epidemic; the statistical analysis showed a marked reduction in the rate of spread during the anticyclonic spell in November, and during this same spell the spread in the Worcester epidemic was not great and was limited to a radius of 4 km. On another occasion (during the final phase in April) a group of outbreaks could be attributed to spread from a nearby source during an anticyclonic spell. In both the latter examples the outbreaks occurred in clusters rather than plumes; this would be expected from spread in light and variable winds characteristic of anticyclonic weather.

When precipitation falls through a plume it increases the number of 4–20 μ diameter particles deposited and neutralizes the dilution due to vertical dispersal. We would therefore expect a greater amount of deposition during rainy than during dry weather, especially beyond about 4 km., where dry deposition appears to be small; this is supported by the evidence we have presented. If the precipitation is intermittent, the deposition will be correspondingly greater where the precipitation does fall because particles will not have been washed out previously; on such occasions, especially with strong winds maintaining concentrations over longer distances, some isolated distant spread can be expected. It thus appears likely that the most distant spread must be associated with strong winds and intermittent rain; the limited evidence available to us supports this. Snow is more efficient than rain at washing out particles; the peaks in the rate of spread (Fig. 6) associated with the periods of snow would agree with this.

The precipitation will deposit particles on grazing and fodder crops (Kindyyakov, 1940), and will also produce a ground level aerosol capable of blowing into buildings. Dry deposition will have similar effects. The virus then has to come into contact with susceptible animals; if it does not, then no outbreaks will occur. The number of outbreaks depends very much on precisely where the virus happens to fall, and may not be closely related to the amount of virus released.

It is not clear whether the deposition on the ground or the ground-level aerosol is the more likely to produce infection, but it would have been expected that housed stock would have been at least partially protected from infection. However, the

evidence suggests that no significant protection was afforded by housing the animals.

Termination of the epidemic

Although much of the behaviour of the epidemic has been accounted for in terms of the weather, several features remain unexplained, the most important being that, after the peak, the epidemic appeared to be less controlled by the weather and continued to decrease in intensity even under what appeared to be suitable weather conditions for a high rate of spread. Previous epidemics also showed these characteristics (Smith & Hugh-Jones, 1969), and it is necessary to look for an explanation. Several suggestions may be made:

(*a*) By analogy with the spread of an infectious disease in a limited population, the decreasing availability of susceptible animals through slaughter, which is not dissimilar in its effect to the development of immunity, and the expansion of the infected area into less densely stocked regions must certainly play a part in reducing the rate of spread during the course of an epidemic. This depopulation occurred in discrete areas, which would still have received virus but, without susceptible stock, would be unable either to demonstrate its reception or to disseminate it further. The total depopulation in Cheshire was about 30%. The continued appearance of disease about later outbreaks would suggest that the development of immunity, without apparent disease, does not occur or is easily overcome. The importance of this hypothesis has still to be assessed.

(*b*) Housing. It has been suggested that a smaller quantity of virus is taken from a housed infected herd than from a similar number of grazing animals. As was seen in Fig. 7, over 60% of infected animals were grazing during the early part of the epidemic, but the proportion decreased steadily until the fifth week, from which time only around 30% were grazing. It is suggested that this played a part in lowering the rate of spread after mid-Nov. It also offers an explanation of why the rate of spread was closely correlated with wind speed only during the early part of the epidemic—variations in outdoor wind speeds have comparatively little influence on indoor airflow. However, it should be borne in mind that the bringing together of animals will favour increased spread within the group and thus produce a more intense source; also that cow-houses are not normally hermetically sealed, and concentrated clouds of virus may be released from an infected cow-house when doors and windows are opened.

(*c*) Slaughter. It is obvious that the sooner an infected herd is slaughtered, the less spread there will be from it. It is not simply the slaughter of animals with clinical symptoms that is important; if slaughter is delayed then many of the apparently healthy animals incubating disease as a result of spread within the group may reach their period of greatest emission. It has been seen (Fig. 8) that up to 26 Nov. more herds were being slaughtered the day after confirmation than on the same day, and a number were left for more than 1 day; after that date the average delay was much reduced. It is suggested that this delay in slaughtering would have contributed to the comparatively high rate of spread during the first half of the epidemic.

Precautions

The conclusion that wind is responsible for most of the spread of foot-and-mouth disease does not make us very optimistic about finding means of limiting the spread. Nevertheless, knowledge of the wind directions which prevailed while farms were infective could be helpful in showing the areas in which search for fresh outbreaks should be concentrated. If the weather was anticyclonic, attention should be concentrated on nearby farms; if it was wet, then the epidemiologist in the field should be prepared for the possibility of outbreaks several tens of kilometres downwind. The housing of all animals in and downwind of an infected area is probably a wise precaution, but while this may help to reduce further spread it is unlikely to prevent those animals themselves from contracting the disease. Any fodder exposed to the weather must be regarded as a potential risk, whether grazed directly or carried into housed animals. However the most effective means of reducing the spread would undoubtedly continue to be the early detection of symptoms and the immediate slaughter of affected and in-contact animals.

Of the many people who have helped in this study we take special pleasure in acknowledging the help and advice of: Mr H. I. Field, lately Director, Central Veterinary Laboratory; the Regional Veterinary Officers responsible for FMD Control Centres during the epidemic; Mr W. Parkinson, D.V.O.; Mrs Doreen Bosbery; Mr Rolland Tinline, Department of Geography, Bristol University; Messrs L. P. Smith and R. P. Rumney (Meteorological Office, Bracknell); Dr D. E Gloyne (Meteorological Office, Edinburgh); Mr W. R. Stansfield (Meteorological Office, R.A.F. Shawbury).

REFERENCES

BURROWS, R. (1968). Excretion of foot and mouth disease virus prior to the development of lesions. *Veterinary Record* **82**, 385–7.

CHAMBERLAIN, A. C. (1955). *Atomic Energy Research Establishment, Report No.* HP/R 1261.

FOGEDBY, E. G., MALMQUIST, W. A., OSTEEN, O. L. & JOHNSON, M. L. (1960). Air-borne transmission of foot and mouth disease virus. *Nordisk Veterinaermedicin* **12**, 490–8.

HARDY, A. C. & MILNE, P. S. (1938). Aerial drift of insects. *Nature, London* **141**, 602–3.

HENDERSON, R. J. (1969). The outbreak of foot-and-mouth disease in Worcestershire. An epidemiological study: with special reference to spread of the disease by wind-carriage of the virus. *Journal of Hygiene* **67**, 21–33.

HURST, G. W. (1968). Foot and mouth disease, the possibility of continental sources of the virus in England in epidemics of October 1967 and several other years. *Veterinary Record* **81**, 610–7.

HYSLOP, N. ST G. (1965*a*). Secretion of foot and mouth disease virus and antibody in the saliva of infected and immunised cattle. *Journal of Comparative Pathology*, **75**, 111–17.

HYSLOP, N. ST G. (1965*b*). Airborne infection with the virus of foot and mouth disease. *Journal of Comparative Pathology* **75**, 119–26.

KINDYYAKOV, V. I. (1940). Investigation on the viability of foot and mouth disease virus outside the body. *Sovyèt Vet.* No. 10, 20–22 (*Veterinary Bulletin, Weybridge* **12**, 532).

MCLEAN, R. C. (1938). Carriers of foot and mouth disease. *Nature, London* **141**, 828.

MEAD, C. J. (1968). Birds as vectors of the foot and mouth virus. *Veterinary Annual* **9**, 70–75.

PASQUILL, F. (1961). The estimation of the deposition of wind-borne material. *Meteorological Magazine, London* **90**, 33–49.

SMITH, L. P. & HUGH-JONES, M. E. (1969). The weather factor in foot and mouth disease epidemics. *Nature, London* **223**, 712–5.

WRIGHT, P. B. (1969). Effects of wind and precipitation on the spread of foot-and-mouth disease. *Weather, London* **24**, 204–13.

APPENDIX

The weather at Pershore meteorological station 6 miles to the south east of the infected area, during the period when most of the spread occurred, can be summarized as follows:

14 Nov. 06 hr.–15 Nov. 18 hr. Downwind bearing 40–80°, mean speed 10 knots. Dry except for slight rain at the beginning and end of the period. (The wind had been on these bearings for the two previous days with traces of rain.)

15 Nov. 18 hr.–15 Nov. 23 hr. Wind veered from 80° to 150° bearing, mean speed 14 knots, 2·1 mm. rain.

15 Nov. 23 hr.–16 Nov. 15 hr. Downwind bearing 150–180°, mean speed 11·2 knots, 0·2 mm. rain during first hour, otherwise dry.

16 Nov. 15 hr.–17 Nov. 21 hr. Wind calm or less than 6 knots from an easterly point.

Thereafter up to 25 Nov. 00 hr. the wind was always from the east, less than 10 knots, often calm with no rain except for a trace on 20 Nov. From 25 Nov. 00 hr.–29 Nov. 03 hr. the wind was from the west, never more than 10 knots and included twelve consecutive calm hours, with 0·1 mm. rain falling about mid-day 25 Nov. Between 03 hr. and 06 hr. on 27 Nov. 1 mm. of rain fell while the wind blew on a downwind bearing of 360°. Thereafter the wind continued to blow from the west.

J. Hyg., Camb. (1970), **68**, 273
Printed in Great Britain

An adhesive surface sampling technique for airborne viruses

By G. THOMAS

Microbiological Research Establishment, Porton Down, Salisbury, Wilts.

(*Received* 20 *January* 1970)

SUMMARY

A mixture of sucrose, glycerol and bovine serum albumin produces a stable coating in a Petri dish which remains adhesive for up to an hour when exposed in a slit sampler. Virus aerosols can be collected on this surface followed by the direct addition of cell cultures to demonstrate the presence of viable virus. The technique is applicable to the Andersen sampler. A modified version of this sampler has been produced with the same particle collection efficiency as the standard Andersen sampler. The plaque counts obtained by the adhesive surface sampling technique are believed to give an indication of the number of particles collected bearing viable virus.

INTRODUCTION

Recovery of viable virus from aerosols of natural origin by several types of aerobiological sampler has proved difficult (Artenstein & Cadigan, 1964; Downie *et al.* 1965; Westwood, Boulter, Bowen & Maber, 1966; Maclean, Bannatyne & Givan, 1967). The use of large volume sampling (10,000–20,000 l./min.) has shown that more frequent recoveries of viable virus could be obtained than had been previously possible (Gerone *et al.* 1966; Artenstein, Miller, Lamson & Brandt, 1968; Winkler, 1968).

The total volume of air sampled by most instruments is mainly limited by loss of water from the sampling medium. Control of this loss was considered most likely where sampling took place onto a surface, e.g. slit sampler (Bourdillon, Lidwell & Thomas, 1941) and the Andersen Sampler (Andersen, 1958), rather than into a liquid. Both of these samplers have been used previously to collect airborne virus. Samples were collected on agar followed by washing (Vlodavets, Gaidamovich & Obukhova, 1960; Jensen, 1964) and on gelatin followed by liquefaction (Dahlgren, Decker & Harstad, 1961; Kuehne & Gochenour, 1961; Couch *et al.* 1965; Gerone *et al.* 1966; Wolfe, Greisemer & Farrell, 1968; Rechsteiner, 1968). It was suspected that loss of viable virus could take place due to inefficient washing. May (1966) showed that bacteria could not be effectively washed off agar. Vlodavets *et al.* (1960) concluded that the apparent low concentration of virus collected by a slit sampler (followed by washing) was explained by partial retention of particles on the agar surface. Jensen (1964) sampled on agar and agar coated with skim milk followed by washing to recover the virus. He found that plain agar alone was only half as efficient as skim milk on agar. It is likely that the skim milk

coating prevented adhesion of virus to the agar, and also limited its diffusion into the agar.

To determine the extent of the loss of viable virus due to washing and diffusion, the gelatin liquefaction and washing methods were examined. The losses were eliminated by the development of an alternative sampling technique which in addition enabled the sampling period to be considerably prolonged.

MATERIALS AND METHODS

Organisms. Bacillus subtilis var *niger*, spores (A.T.C.C. 9372); vaccinia virus, Lister Institute vaccine; polio virus, Sabin attenuated type 1 strain L Sc 2ab; Semliki Forest virus (Smithburn & Haddow, 1944).

Cell lines. Mainly a HeLa cell line (Appleyard & Westwood, 1964) was used but other lines were also tested including eight animal kidney cell lines, a guinea pig heart line, chick embryo cells and a Wistar 38 cell line.

Methods. Suspensions of spores ($7{\cdot}5 \times 10^3$/ml.) in phosphate buffer manucol agar with 0·1 % antifoam, or viruses (2×10^5–1×10^8 pfu/ml.) in 199 medium with antifoam were sprayed in an aerosol apparatus (Henderson, 1952) at 55 % R.H., 23° C. The aerosol could be suspended in a drum (Goldberg, Watkins, Boerke & Chatingny, 1958) or led into an Andersen or slit sampler, both sampling at 1 ft.^{3}/min. (28·3 l.). Control samples were taken from the spray tube with Porton impingers, operating at 0·406 ft.^{3}/min. (11·5 l.), containing phosphate buffer or 199 medium mixtures as appropriate. Vaccinia virus was assayed on monolayers and the polio virus and Semliki Forest virus in suspended cell cultures in Noble agar.

PRELIMINARY INVESTIGATIONS

Liquefaction of 12 % gelatin, to recover vaccinia virus, followed by addition to HeLa cells resulted in disturbed monolayers. This was probably a toxic effect of the gelatin used and has been observed by other workers (Artenstein *et al.* 1968).

Washing methods were used to recover vaccinia and polio viruses from agar surfaces. Equal volumes of virus suspension were spread on agar and added to HeLa cell suspensions as controls. The agar surfaces were irrigated thoroughly with nutrient medium to recover virus and the washings mixed with HeLa cells.

Plaque counts obtained with vaccinia and polio virus are given in Table 1. Recovery of virus, despite thorough washing, was erratic and, on average, less than 50 % could be removed from the agar surface. With polio virus, HeLa cell suspensions were added to the agar surfaces both after and before washing. The resulting plaque counts (Table 1; Polio 1*c*) showed that after washing a good deal of virus was still present on the surface of the agar, while addition of HeLa cells 10 min. after spreading the virus suspension showed that much of the virus appeared to have diffused into the agar (Table 1; Polio 2*c*). This was confirmed by spreading polio virus on the surface of agar containing suspended HeLa cells with subsequent plaque formation. The ability of the virus to diffuse is of course a prerequisite of plaque formation in the agar suspended tissue cell technique.

When direct sampling on prepared cell cultures was attempted it was found that monolayers, under liquid medium, were quickly disrupted when exposed in a slit sampler. Exposure of suspended cell cultures in agar resulted in the death of the cells near the surface after 15 min. A Semliki Forest virus aerosol was sampled for periods up to 30 min. on suspended cell cultures with a slit sampler. Plaque formation was not visible after more than 7 min. sampling.

Gelatin liquefaction, washing methods and direct sampling on cell cultures were found therefore to have severe limitations. Prolongation of the sampling period

Table 1. *Recovery of vaccinia and polio viruses from agar surfaces*

Virus	Samples	Plaque counts per sample					Mean counts
Vaccinia	(*a*) Controls	22	30	27	20		25
	(*b*) Agar surface washings	4 8	5 15	16 12	19 6		11
Polio 1	(*a*) Controls	34 52	32 41	36 62	33 51	32 48	42
	(*b*) Agar surface washings	23 11	12 15	26 10	25 11	25 5	17
	(*c*) Cells added to washed agar	9 11	10 11	5 16	5 31	7 35	14
Polio 2	(*a*) Controls	90 54	88 60	60 44	67 42	68 60	63
	(*b*) Agar surface washings	29 10	20 10	6 14	3 20	5 17	13
	(*c*) Cells added without washing	7 8	9 16	8 9	10 26	17 —	12

— = No sample.

to sample larger volumes of air required control of evaporation. This necessitated the development of a suitable medium to produce a sampling surface resistant to water loss on which to collect airborne virus particles. If tissue culture cells could then be added directly to this surface, to demonstrate viable virus without further manipulation of the sample, the losses occurring in the recovery methods described above would be avoided.

Development of an adhesive sampling surface

The use of an adhesive surface to collect airborne virus was designed to serve two purposes. First, by reducing water loss it would enable much larger volumes of air to be sampled using long sampling periods (30–60 min.), and second by eliminating losses inherent in other sampling methods it would enhance the recovery of small amounts of viable virus.

The main requirements of such a sampling surface were that it should remain adhesive for the whole of the sampling period, retaining particles produced by

shattering on impact (Davies, Aylward & Lacey, 1951), and it should be compatible with viruses and cell cultures.

Twenty-one substances and mixtures were examined for (*a*) their ability to remain adhesive for 1 hr. on a slit sampler, (*b*) water solubility at 37° C. on completion of the sampling period and (*c*) compatibility with the viruses and cell lines given in Materials and Methods. The substances tested included calf serum, bovine serum albumin, gelatin, egg albumin, carboxymethyl cellulose, polyethylene glycol, dextran, glycerol, hydrolysed starch, sucrose, skim milk and various mixtures of

Table 2. *Effect of the S.G.B. surface on plaque formation by vaccinia and polio virus*

Virus	Samples	Plaque counts per sample					Mean counts
Vaccinia	Controls	43	40	43	39	36	40
	S.G.B.	38	40	34	39	37	38
Polio 1	Controls	14	8	16	11	13	12
	S.G.B.	15	23	17	11	12	16
Polio 2	Controls	37	38	39	39	34	37
	S.G.B.	29	33	38	40	50	38
Polio 3	Controls	140	134	162	132	154	144
	S.G.B.	155	146	140	174	151	153

Table 3. *Effect of exposure in a slit sampler on plaque formation with the S.G.B. mixture*

Virus	Sample	Length of exposure (min.)	Plaque counts per sample					Mean counts
Vaccinia	Control	0	43	40	43	39	36	40
	Slit sampler	6	36	33	39	40	32	36
Polio 1	Control	0	28	26	36	32	30	30
	Slit sampler	5	20	33	34	20	36	29
		10	10	29	47	38	20	29
		20	28	36	—	—	—	32
		30	26	30	—	—	—	28
Polio 2	Control	0	56	64	62	50	62	59
	Slit sampler	5	63	71	77	84	94	78
		10	96	85	98	89	70	88
		20	80	66	—	—	—	73
		30	77	51	—	—	—	64

— = No sample.

the foregoing. A mixture of equal parts of a saturated solution of sucrose and glycerol with 0·1% of 10% bovine serum albumin (S.G.B. mixture) eventually proved satisfactory. An adhesive surface which fulfilled all the requirements was produced by spreading 0·2 ml. of this mixture in a Petri dish. It remained adhesive for up to 1 hr. exposure in a slit sampler. Cell suspensions added to these exposed plates formed satisfactory monolayers.

Quantitative tests with vaccinia and polio virus, to examine the effect of the

S.G.B. surface on plaque formation (Table 2), did not indicate any inhibitory action. Suspensions of both viruses were spread on prepared S.G.B. surfaces and then the plates were exposed for varying periods in a slit sampler. The results (Table 3) did not show any evidence of inhibition of plaque formation. Growth of common airborne bacteria and fungal spores was prevented by the presence of antibiotics and an antifungal agent, amphotericin B (0·0025 mg./ml.) in the nutrient medium.

AEROSOL EXPERIMENTS

Bacterial spore aerosols

In order to establish the comparative sampling efficiency of the slit with the impinger and Andersen samplers preliminary tests were carried out with the bacterial spore in the aerosol apparatus. The results are given in Table 4. Break up of particles in the impinger would of course result in higher colony counts in comparison with the slit sampler. The impinger and the Andersen samplers have higher collection efficiencies than the slit sampler for particles of 1 μ and less.

Table 4. *Comparison of the collection efficiency of the slit sampler with the impinger and Andersen samplers using bacterial spore aerosols and agar plates*

Sampler	Time (min.)	Volume sampled (ft.^{3})	Colony counts per sample					Mean count per ft.^{3}	C.E. (%)
Slit	1	1	209	270	—	—	—	239	60
Imp.		0·406	162	158	—	—	—	397	
Slit	1	1	63	66	57	47	51	56	64
Imp.		0·406	36	32	40	38	34	88	
Slit	1	1	30	30	28	28	38	31	63
And.		1	46	53	52	43	51	49	

Slit, Slit sampler; Imp., Impinger; And., Andersen sampler; C.E., Collection efficiency.

As can be seen from Table 4, very similar percentage collection efficiencies were obtained in the comparisons of the slit sampler with the impinger (60 % and 64 %) and the Andersen sampler (63 %).

Erhlich, Miller & Idoine (1966) compared the collection efficiencies of the slit sampler and the impinger using the same bacterial spore. Particles collected by the slit sampler were recovered by washing. An average figure of 50 % was obtained for the comparative collection efficiency. No account was taken of losses which occur during washing to recover the spores. Each Petri dish in the standard Andersen sampler normally contains 27 ml. of agar to bring the sampling surface to the correct height (0·1 in.) below the orifice plates. In order to use the adhesive surface technique with this sampler, glass disks coated with the S.G.B. mixture were tried initially to replace the agar. However, they proved generally unsuitable for monolayer work. Consequently a modified Andersen sampler was constructed in which the orifice plates were lowered to the requisite distance above the sampling plates coated with the S.G.B. mixture.

The sampling characteristics of the modified Andersen sampler were compared with the standard version using spore aerosols. Table 5 gives the results showing that there were no significant differences in collection characteristics of the two versions of the sampler.

Table 5. *Comparison of the standard and modified Andersen samplers using a bacterial spore aerosol*

	Volume sampled (ft.³)	Colony counts for stage number 2	3	4	5	6	Mean total counts
Standard Andersen sampler	1	2	0	3	19	22	50
		0	0	8	15	30	
Modified Andersen sampler	1	0	0	7	12	27	51
		0	0	1	18	42	
		0	0	2	13	31	

The plates in the modified sampler contained a thin layer of agar on which colonies formed satisfactorily. Stage 1 was not used in either sampler.

Table 6. *Comparison of collection efficiency of the slit sampler (using the S.G.B. surface) with the impinger, sampling aerosols of vaccinia and polio viruses*

Sampler	Virus	Time (min.)	Volume sampled (ft.³)	Plaque counts per sample					Mean counts per ft³	C.E. (%)
Slit	Vaccinia	½	0·5	100	112	146	118	142	271	52
				212	94	148	106	178		
Imp.			0·203	11	10	9	14	11	521	
				10	11	9	12	9		
Slit	Vaccinia	½	0·5	160	170	153	158	159	320	65
Imp.			0·203	7	10	10	11	12	492	
Slit	Vaccinia	10	10	117	120	127	—	—	12	57
Imp.			4·06	8	9	8	—	—	21	
Slit	Polio	½	0·5	126	116	120	—	—	240	61
Imp.			0·203	9	8	7	—	—	392	

Slit, Slit sampler; Imp., Impinger sampler; C.E., Collection efficiency.

Note. The plaque counts given for the impinger samples are the results of assay of 1 ml. out of the 10 ml. of sampling fluid in each impinger.

Virus aerosols

Vaccinia virus was sampled with the slit sampler on S.G.B. mixture plates at different aerosol concentrations. Half-minute samples were taken at the higher concentrations and 10 min. samples at the lower. Similar samples were taken with impingers. HeLa cells were poured directly onto the S.G.B. surface after sampling to form monolayers while the impinger samples were assayed by mixing 1 ml. amounts of the sampling liquid with HeLa cells. The results given in Table 6 showed that the comparative collection efficiency varied between 52 and 65 %. Polio virus was used in a similar experiment. HeLa cells suspended in agar were added to the adhesive surface on completion of sampling. The plaque counts

obtained are given in Table 6. A comparative collection efficiency of 61 % was obtained.

A vaccinia virus aerosol was sampled with the slit sampler and the modified Andersen sampler with the results given in Table 7. The comparative collection efficiency was 51 %. Impinger samples were also taken during this experiment, giving a comparative collection efficiency of 78 % when compared with the Andersen sampler.

Table 7. *Comparison of the collection efficiency of the slit sampler and the impinger with the modified Andersen sampler using a vaccinia virus aerosol*

Sampler	Volume sampled (ft.³)	Plaque counts per sample						Mean counts per ft.³	C.E. (%)
Slit	0·5	160	170	153	158	159		320	51
Impinger	0·203	7	10	11	11	12		492	78
		Plaque counts for stage no.							
		2	3	4	5	6	Totals		
Modified Andersen	0·5	2	9	15	14	238	278	627	
		1	9	15	14	310	349		

Note. The plaque counts given for the impinger samples are the results of assay of 1 ml. out of the 10 ml. of sampling fluid in each impinger. S.G.B. plates were used in the slit and modified Andersen samplers. Stage 1 was not used in the Andersen sampler. C.E. = Collection efficiency.

DISCUSSION

The collection efficiency of the slit sampler, using the adhesive surface method for sampling virus aerosols was 52–65 % when compared with the impinger. This result is very similar to that obtained with the tracer spore aerosol (60–64 %). Colony counts obtained with bacteria in a slit sampler indicate the number of particles bearing viable organisms in the aerosol, while the disruptive effects of the sampling process in the impinger give rise to colony counts which relate to the number of viable bacteria in the aerosol. Since the comparative collection efficiency of the slit sampler was very similar for both the spore and the virus aerosols it is possible the plaque counts obtained with the slit sampler give an indication of the number of particles bearing viable virus. A similar conclusion is applicable to the results obtained with the Andersen sampler.

The smallest particles in the bacterial spore aerosol detectable on the last stage of the Andersen sampler could not be smaller than the size of the individual spores, i.e. about 1 μ. Smaller particles are however produced by the Collison spray and through drying of the aerosol. When viruses are used these submicron particles can carry viable virus and hence are detectable when collected on the last stage. This is shown by comparison of the percentage distribution recoveries calculated from the modified Andersen sampler results (Tables 5 and 7) obtained with the spore and virus aerosols. An average of 55 % of the spore aerosol was collected on the last (6th) stage compared with 70 % of the virus aerosol. This accounts for the

apparent fall in the collection efficiency of the slit sampler when compared with the Andersen sampler from 63 % of spore aerosols to 51 % with virus aerosols. The difference is due to the superior collection efficiency of the Andersen sampler for submicron particles compared with the slit sampler. When the impinger was compared with the modified Andersen sampler (Table 7) it had a comparative sampling efficiency of 78 %. Jensen (1964) also found the Andersen sampler was more efficient than glass impingers for sampling virus aerosols.

Guerin & Mitchell (1964) sampling virus aerosols with an Andersen sampler used only 5 ml. of gelatin in each dish instead of the normal 27 ml. This would be likely to alter the sampling characteristics mainly in the direction of loss of smaller particles. The percentage distribution recoveries they obtained for an aerosol agent in a drum were 26, 31 and 31 % for stages 4, 5 and 6 respectively. In contrast, the present author found in a similar drum experiment that 81 % of the virus aerosol collected was retained on the last stage of the sampler.

Couch *et al.* (1965) sampled virus aerosols, from a Collison spray, on gelatin layered on agar (total volume 27 ml.), in an Andersen sampler from aerosol apparatus similar to that used by the author. They observed that 85 % of the particles collected were less than 1 μ in size and that more than 90 % of the virus was retained on the lower three stages. This finding is closely similar to the figures given in Table 7, where 95 % of the virus was collected on the lower three stages. The distribution of virus on these three stages was however different from those reported by Couch *et al.*:

Couch *et al.* (1965)	This report
13 %	4·5 %
68 %	20·6 %
10 %	70·50 %

Couch *et al.* estimated the proportion of aerosolized material retained on each stage and found that 54 % of the total volume collected was in the particle size range 1–2 μ, i.e. on the penultimate stage. After sampling the gelatin was liquefied, serially diluted and inoculated into roller tube cultures. These manipulations tend to cause break up of the collected aerosol particles disseminating the virus. Consequently the plaque counts obtained by this method indicate the amount of viable virus collected on each stage. The larger volume of aerosolized material and hence greater proportion of virus collected on the penultimate stage is shown by the higher *percentage recovery* on this stage calculated from the plaque counts. In contrast the adhesive surface sampling method, since it causes little disturbance of the sample, gives an indication of the number of particles bearing viable virus collected on each stage. As Couch *et al.* found, 85 % of the particles were less than 1 μ, many carrying viable virus, and so, with the method described in this report, would result in a high percentage recovery on the last stage.

The adhesive surface sampling method is a simple technique which enables larger volumes of air to be sampled by the slit and Andersen samplers. Direct addition of cell cultures to the sample without further manipulation aids the

detection of small amounts of viable virus which might be lost by the use of the other methods described. Survival of the viruses used in these investigations on the adhesive surface during prolonged sampling periods was good. But the method has yet to be tried with less robust viruses. The large volume samplers which have been successfully used for the recovery of virus from natural aerosols give information on the amount of viable virus collected. The adhesive surface method indicates the number of particles bearing viable virus and also, with the Andersen sampler, their size distribution.

REFERENCES

ANDERSEN, A. (1958). A new sampler for the collection, sizing and enumeration of viable airborne particles. *Journal of Bacteriology* **76**, 471.

APPLEYARD, G. & WESTWOOD, J. C. N. (1964). The growth of rabbit pox virus in tissue cultures. *Journal of General Microbiology* **37**, 391.

ARTENSTEIN, M. S. & CADIGAN, F. C. JR. (1964). Air sampling in viral respiratory disease. *Archives of Environmental Health* **9**, 58.

ARTENSTEIN, M. S., MILLER, W. S., LAMSON, T. H. & BRANDT, B. L. (1968). Large volume air sampling for meningococci and adenoviruses. *American Journal of Epidemiology* **87**, 567.

BOURDILLON, R. B., LIDWELL, O. M. & THOMAS, J. C. (1941). A slit sampler for collecting and counting airborne bacteria. *Journal of Hygiene* **41**, 197.

COUCH, R. B., GERONE, P. J., GATE, T. R., GRIFFITH, W. R., ALLING, D. W. & KNIGHT, V. (1965). Preparation and properties of a small particle aerosol of Coxsackie A 21. *Proceedings of the Society for Experimental Biology and Medicine* **118**, 818.

DAHLGREN, C. M., DECKER, H. M. & HARSTAD, J. B. (1961). A slit sampler for collecting T3 bacteriophage and Venezuelan equine encephalomyelitis virus. 1. Studies with T3 bacteriophage. *Applied Microbiology* **9**, 103.

DAVIES, C. N., AYLWARD, M. S. & LACEY, D. (1951). Impingement of dust from air jets. *American Medical Association Archives of Industrial Hygiene* **4**, 354.

DOWNIE, A. W., MEIKLEJOHN, M., VINCENT, L. ST, RAO, A. R., SUNDARA BABU, B. V. & KEMPE, C. H. (1965). The recovery of smallpox from patients and their environment in smallpox hospitals. *Bulletin of the World Health Organization* **33**, 615.

ERHLICH, R., MILLER, S. & IDOINE, L. S. (1966). Use of slit sampler in comparison with impinger. *Applied Microbiology* **14**, 328.

GERONE, P. J., COUCH, R. B., GARRETT, V. K., DOUGLAS, R. G., DERRENBACKER, E. B. & KNIGHT, V. (1966). Assessment of experimental and natural aerosols. *Bacteriological Reviews* **30**, 576.

GOLDBERG, L. J., WATKINS, H. M. S., BOERKE, E. E. & CHATINGNY, M. A. (1958). The use of a rotating drum in the study of aerosols over extended periods of time. *American Journal of Hygiene* **68**, 85.

GUERIN, L. F. & MITCHELL, C. A. (1964). A method for determining the concentration of airborne virus and sizing droplet nuclei containing the agent. *Canadian Journal of Comparative Medicine* **28**, 283.

HENDERSON, D. W. (1952). An apparatus for the study of airborne infections. *Journal of Hygiene* **50**, 53.

JENSEN, M. M. (1964). Inactivation of airborne virus by U.V. irradiation. *Applied Microbiology* **12**, 418.

KUEHNE, W. R. & GOCHENOUR, W. S. (1961). A slit sampler for collecting T3 bacteriophage and Venezuelan equine encephalomyelitis virus. 2. Studies with Venezuelan equine encephalomyelitis virus. *Applied Microbiology* **9**, 105.

MACLEAN, D. M., BANNATYNE, R. M. & GIVAN, K. F. (1967). Myxovirus dissemination by air. *Canadian Medical Association Journal* **96**, 1449.

MAY, K. R. (1966). A multistage liquid impinger. *Bacteriological Reviews* **30**, 559.

RECHSTEINER, J. (1968). *Phenomena of Decay in Respiratory Syncytial Virus*. Rotterdam: Bronder-Offset.

SMITHBURN, K. C. & HADDOW, A. J. (1944). Semliki Forest virus. *Journal of Immunology* **49**, 141.

VLODAVETS, V. V., GAIDAMOVICH, S. Y. & OBUKHOVA, V. R. (1960). Methods of trapping influenza virus in the droplet phase of an aerosol. *Problems of Virology* **5**, 728.

WESTWOOD, J. C. N., BOULTER, E. A., BOWEN, E. T. & MABER, H. B. (1966). Experimental respiratory infection with pox viruses. 1. Clinical, virological and epidemiological studies. *British Journal of Experimental Pathology* **48**, 453.

WINKLER, W. G. (1968). Airborne rabies virus isolation. *Bulletin of the Wildlife Diseases Association* **4**, 37.

WOLFE, L. G., GREISEMER, R. A. & FARRELL, R. L. (1968). Experimental transmission of Yaba virus in monkeys. *Journal of the National Cancer Institute* **41**, 1175.

J. Hyg., Camb. (1970), **68**, 283
With 1 plate
Printed in Great Britain

The use of cough/nasal swabs in the rapid diagnosis of respiratory syncytial virus infection by the fluorescent antibody technique

By JOYCE McQUILLIN, P. S. GARDNER and PATRICIA M. STURDY

Department of Virology, Royal Victoria Infirmary, and University of Newcastle upon Tyne, Newcastle upon Tyne

(*Received* 22 *January* 1970)

SUMMARY

Thirty-five consecutive infants admitted into hospital in Newcastle upon Tyne with acute respiratory disease had cough/nasal swabs and nasopharyngeal secretions taken. Both types of specimens were examined by the fluorescent antibody technique for respiratory syncytial virus; isolation techniques were also used. Twenty-eight specimens of nasopharyngeal secretion were positive, as were 26 of the corresponding cough/nasal swab preparations. Respiratory syncytial virus was isolated from all but one.

Sixteen consecutive children who were only suitable for examination by cough/nasal swab preparations were also investigated by isolation and fluorescent antibody techniques for respiratory syncytial virus. Respiratory syncytial virus was isolated from eight, seven of whom were positive by the fluorescent antibody technique. The use of cough/nasal swab preparations stained by the fluorescent antibody technique, although not as efficient as nasopharyngeal secretions, may have a place in the rapid diagnosis of respiratory virus infection in older children and children in general practice. The importance of rapid diagnosis for respiratory virus infection in relationship to antiviral therapy was also discussed.

INTRODUCTION

The rapid diagnosis of acute respiratory infection in childhood has made considerable progress over the last few years along two main lines. Some workers have examined nasopharyngeal secretions by electron microscopy and have attempted to make provisional diagnoses on the appearance of virus particles present (Doane *et al.* 1967; Joncas *et al.* 1969; Doane, Anderson, Zbitnew & Rhodes, 1969). Others have applied fluorescent antibody techniques to secretions from the nasopharynx; a rapid diagnosis for respiratory syncytial (R.S.) virus (McQuillin & Gardner, 1968; Gardner & McQuillin, 1968*a*), and for influenza (Hers, van der Kuip & Masurel, 1968) has been investigated in this way.

Collection of nasopharyngeal secretions is a technique only suitable for use with children who are less than 2 years of age and who are in hospital. It is not suitable for use outside hospital. Portable suction equipment is required unless each ward has suitable suction facilities. Claims have been made for a successful fluorescent

antibody technique for the rapid diagnosis of R.S. virus infection by smearing throat swabs directly on to slides (Gray, MacFarlane & Sommerville, 1968) but, on analysis, these results were not considered sufficiently reliable (Miller & Taylor, 1968; Gardner & McQuillin, 1968*b*). In 1968 McQuillin & Gardner had tried this method and found it unsuitable because of the absence of intact cells and the large amount of debris.

This communication describes a revised method for studying cells obtained from cough/nasal swabs after eluting, washing, concentrating and fixing them on slides, so producing preparations of intact cells suitable for staining by the fluorescent antibody technique. The results of staining cells taken on cough/nasal swabs in this way by the fluorescent antibody technique for R.S. virus have been compared with those of staining cells in materials taken by suction from the nasopharynx. Haire (1968), too, has recently reported the successful use of throat swabs stained by a similar technique for the rapid diagnosis of infection with rubella virus.

MATERIALS AND METHODS

Thirty-five cough/nasal swab preparations were taken from consecutive children who were also suitable patients for aspiration of nasopharyngeal secretions; in addition, 16 consecutive patients who were unsuitable for aspiration only had cough/nasal swabs taken. All specimens were examined both by isolation and by the fluorescent antibody techniques.

Nasopharyngeal secretions

The technique of taking and preparing nasopharyngeal secretions has been described fully elsewhere (Sturdy, McQuillin & Gardner, 1969).

Cough/nasal swabs

The patient's pharynx was swabbed thoroughly until he gagged and coughed onto the swab; in addition, a nasal swab was taken. The swabs were then broken in the same bijoux bottle of transport medium (Hanks' medium with 0·2 % bovine albumen and antibiotics) and the specimen transported to the laboratory on melting ice.

The medium in the specimen bottle was pipetted over the swabs until the secretions adhering to them were suspended. The medium was transferred to another bottle and the original bottle containing the swabs was kept at a temperature of 4°C. The medium was centrifuged at 500 rev./min. for 10 min. in a refrigerated centrifuge to deposit the cells. The supernatant fluid was removed and returned to the original bottle (kept at a temperature of 4°C.), to be used for subsequent virus isolations. The cell deposit was re-suspended in phosphate buffered saline to wash the cells and again centrifuged at 500 rev./min. for 10 min. The resultant washed cell deposit was re-suspended in a small volume of phosphate buffered saline, sufficient to make two or three slides with three spots of cell deposit on each, the number of slides depending on the amount of cell deposit available. The cell spots were allowed to dry in air, then fixed in acetone at 4°C. for 10 min.;

they were then ready for staining by the fluorescent antibody technique, as described in previous publications (McQuillin & Gardner, 1968; Gardner & McQuillin, 1968*a*).

The isolation and identification of viruses

The supernatant fluids from cough/nasal swab specimens stored at 4°C. were used for attempted virus isolations. This material was inoculated in 0·2 ml. volumes on two tubes each of 'Bristol' HeLa, HEp 2 and rhesus monkey kidney cells and on one tube of W.I. 38 cells. The preparation of nasopharyngeal secretions has been described in previous publications, as has the isolation of viruses from them; the identification of virus strains has also been previously described (McQuillin & Gardner, 1968; Sturdy *et al.* 1969).

Fluorescent antibody methods for the identification of viruses

The fluorescent antibody technique used in this study to identify R.S. virus in exfoliated cells from the nasopharynx and to confirm the identity of iolations of R.S. virus were the same as those described elsewhere (McQuillin & Gardner, 1968; Gardner & McQuillin, 1968*a*).

All specimens of cough/nasal swabs and nasopharyngeal secretions stained by the fluorescent antibody technique were examined by two of the authors; the cough/nasal swabs were also re-examined under coded numbers without knowledge of the results of the previous examinations.

RESULTS

Surprisingly large numbers of intact cells were found on slides prepared from cough/nasal swab specimens, but these were mainly squamous cells and a great deal of debris was always present, some of which gave a uniform green non-particulate fluorescence and some a bright orange fluorescence, quite different from the specific particulate apple-green fluorescence of R.S. virus. Infected cells exhibiting specific fluorescence in cough/nasal swab preparations appeared to be small ones, probably macrophages and cells shed from the respiratory tract; occasionally, ciliated cells were observed. All these cells appeared to be the same type as those seen to be infected in nasopharyngeal secretions.

In this present series, infected cells were found in all the positively stained nasopharyngeal secretions within minutes of examining them under the fluorescent microscope. On the other hand, infected cells in cough/nasal swab preparations were very scanty and an intensive search, taking in many cases up to 20 min., was required to locate them. The fluorescence of positive cells in cough/nasal swab preparations was of lower intensity and it was more difficult to find an intact cell containing fluorescent particles or inclusions, which we have stipulated as being one of our basic requirements for making a positive diagnosis; in many cases, the cells exhibiting positive fluorescence appeared to be damaged. However, in all those cases except two, where the nasopharyngeal secretions were positive, cough/nasal swab preparations were also positive.

Table 1. *Results of examination of cough/nasal swabs and nasopharyngeal secretions stained by fluorescent antibody technique for respiratory syncytial virus, and virus isolation on tissue culture*

Patient	Diagnosis	Age (months)	Specimen	Result of F.A.	Result of Virus isolation	Day of isolation
M. M.	Bronchiolitis	1¼	C/swab	+	R.S.V.	11
			N.P.S.	+	R.S.V.	7
P. R.	Ac. bronchitis	4	C/swab	–	P. inf. 3	.
			N.P.S.	–	P. inf. 3	.
P. K.	Bronchiolitis	3	C/swab	+	R.S.V.	6
			N.P.S.	+	R.S.V.	7
M. A.	Ac. bronchitis	11	C/swab	+	R.S.V.	6
			N.P.S.	+	R.S.V.	6
G. C.	Croup	36	C/swab	–	Nil	.
			N.P.S.	–	Nil	.
J. S.	Pertussis	6	C/swab	–	Adeno 5	15
			N.P.S.	–	Adeno 5	11
G. W.	Ac. bronchitis	2½	C/swab	+	R.S.V.	13
			N.P.S.	+	R.S.V.	6
R. S.	Bronchiolitis	2½	C/swab	+	R.S.V.	13
			N.P.S.	+	R.S.V.	4
A. R.	Bronchitis	21	C/swab	–	Nil	.
			N.P.S.	–	Nil	.
S. R.	Bronchitis	7	C/swab	+	R.S.V.	16
			N.P.S.	+	R.S.V.	9
S. T.	Bronchitis	1½	C/swab	+	Nil	.
			N.P.S.	+	Nil	.
S. W.	Bronchitis	10	C/swab	+	R.S.V.	14
			N.P.S.	+	R.S.V.	8
K. C.	Bronchitis	10	C/swab	+	R.S.V.	17
			N.P.S.	+	R.S.V.	14
N. O.	Bronchitis	1¼	C/swab	–	P. inf. 3	.
			N.P.S.	–	P. inf. 3	.
S. H.	Bronchiolitis	9	C/swab	+	R.S.V.	8
			N.P.S.	+	R.S.V.	6
H. S.	Bronchiolitis	3	C/swab	+	R.S.V.	6
			N.P.S.	+	R.S.V.	6
H. W.	Bronchiolitis	7	C/swab	–	Nil	.
			N.P.S.	–	Nil	.
P. T.	Bronchiolitis	2	C/swab	+	R.S.V.	14
			N.P.S.	+	R.S.V.	6
A. B.	Bronchiolitis	4	C/swab	–	Nil	.
			N.P.S.	–	Nil	.
L. N.	Coryza	1	C/swab	+	R.S.V.	14
			N.P.S.	+	R.S.V.	12
K. P.	Bronchiolitis	5	C/swab	+	R.S.V.	13
			N.P.S.	+	R.S.V.	11
D. R.	Bronchiolitis	7	C/swab	+	R.S.V.	10
			N.P.S.	+	R.S.V.	4
D. K.	Bronchiolitis	1¼	C/swab	+	R.S.V.	11
			N.P.S.	+	R.S.V.	12
G. H.	Bronchiolitis	2½	C/swab	+	R.S.V.	15
			N.P.S.	+	R.S.V.	4
S. B.	Bronchiolitis	2¼	C/swab	+	R.S.V.	11
			N.P.S.	+	R.S.V.	4

Table 1. (*cont.*)

Patient	Diagnosis	Age (months)	Specimen	Result of F.A.	Result of Virus isolation	Day of isolation
S. M.	Coryza	¾	C/swab	+	R.S.V.	10
			N.P.S.	+	R.S.V.	9
W. I.	Bronchiolitis	3	C/swab	+	R.S.V.	14
			N.P.S.	+	R.S.V.	7
P. M.	Bronchiolitis	1	C/swab	+	R.S.V.	11
			N.P.S.	+	R.S.V.	9
T. F.	Bronchitis	3	C/swab	−	R.S.V.	11
			N.P.S.	+	R.S.V.	11
C. C.	Bronchiolitis	3	C/swab	−	R.S.V.	18
			N.P.S.	+	R.S.V.	21
S. H.	Wheezy bronchitis	4	C/swab	+	R.S.V.	8
			N.P.S.	+	*Polio	
R. R.	Bronchiolitis	15	C/swab	+	R.S.V.	9
			N.P.S.	+	R.S.V.	9
S. R.	Croup	21	C/swab	+	R.S.V.	18
			N.P.S.	+	R.S.V.	10
T. R.	Wheezy bronchitis	2½	C/swab	+	R.S.V.	15
			N.P.S.	+	R.S.V.	13
M. B.	Wheezy bronchitis	9	C/swab	+	R.S.V.	8
			N.P.S.	+	R.S.V.	8

* Oral polio vaccine recently given. Difficulty in isolating R.S.V. except in cough/nasal swab.

F.A. = Fluorescent antibody. C/swab = Cough/nasal swab. N.P.S. = Nasopharyngeal secretion. R.S.V. = Respiratory synctial virus. P. inf. 3 = Parainfluenza virus type 3. Adeno. 5 = Adenovirus type 5.

In those 16 children from whom only cough/nasal swabs were taken, seven were positive by direct fluorescence for R.S. virus; all were confirmed by virus isolation. One R.S. virus isolation was made from a child in this group in whom the cough/nasal swab preparation was negative by the fluorescent antibody technique. Plate 1, figs. 1 and 2 show cells eluted from the cough/nasal swabs of two infants with bronchiolitis and stained by the indirect fluorescent antibody technique for R.S. virus. Plate 1, fig. 1 shows a considerable amount of non-specific fluorescence, but nevertheless specific fluorescence is still detectable in two cells; Pl. 1, fig. 2 shows that occasional cells showing positive fluorescence are as easily detected as they are in nasopharyngeal secretions. Plate 1, fig. 3 shows cells in a nasopharyngeal secretion stained in the same way; there is little background non-specific staining.

Table 1 summarizes the results obtained by examination of the nasopharyngeal secretions and cough/nasal swabs in parallel from our series of infants. Table 2 summarizes the results in those patients from whom only cough/nasal swabs were taken. The results of virus isolations are also included, as is the time taken for identification of R.S. virus in tissue culture. The average time taken for the isolation of R.S. virus from cough/nasal swabs is longer than that from nasopharyngeal secretions and confirms our observations as to the relative scantiness of infected cells in cough/nasal swab preparations and also our previous comparison of cough/nasal swabs and nasopharyngeal secretions (Sturdy *et al.* 1969). Table 3 compares

the efficacy of cough/nasal swab preparations with nasopharyngeal secretions, both stained by the fluorescent antibody technique for R.S. virus. Table 4 compares the efficiency of cough/nasal swab preparations with the standard isolation techniques in those children from whom it was impossible to obtain nasopharyngeal secretions.

Table 2. *A comparison of virus isolation on tissue culture, and the examination of cells on cough/nasal swabs for respiratory syncytial virus by the fluorescent antibody technique*

Patient	Diagnosis	Age	Result of F.A. for R.S.V.	Result of Virus isolation	Day of isolation
B. D.	Pneumonia	2 years	+	R.S.V.	8
W. C.	Pneumonia	4 years	+	R.S.V.	11
S. K.	Pneumonia	4 years	+	R.S.V.	11
A. B.	Febrile convulsions	2 years	+	R.S.V.	5
M. G.	U.R.T.I.* and febrile convulsions	14 months	+	R.S.V.	10
L. H.	U.R.T.I.	5 years	+	R.S.V.	8
L. D.	Bronchitis	2 years	−	—	.
M. B.	U.R.T.I.	5 years	−	—	.
L. H.	Pneumonia	2 years	−	—	.
G. M.	Pneumonia	6 years	+	R.S.V.	15
T. F.	Bronchitis	2½ years	−	—	.
T. C.	U.R.T.I.	5 weeks	−	—	.
J. H.	U.R.T.I.	3 years	−	—	.
B. R.	Bronchitis	20 months	−	—	.
D. J.	Croup	6 years	−	R.S.V.	8
I. M.	Croup	3 years	−	P. inf. 1.	.

* U.R.T.I. = Upper respiratory tract infection. F.A. = Fluorescent antibody. R.S.V. = Respiratory syncytial virus. P. inf. 1 = Parainfluenza virus type 1.

DISCUSSION

It is less distressing and more practicable to take a cough/nasal swab than to aspirate the nasopharynx in children over the age of 1 year. Moreover, cough/nasal swabs can be taken from children of all ages, whether at home or in hospital, whereas nasopharyngeal secretions can only be obtained easily from patients under the age of 2 years in hospital.

There is no doubt that the examination of nasopharyngeal secretions is the method of choice and where possible they should be used, for, not only are the infected cells far more abundant, but the material is also a much better source of virus for isolation procedures (Sturdy *et al.* 1969). Preparations of nasopharyngeal secretions take far less time to examine than the corresponding specimen of cough/nasal swab. However, in the very young infant or the older child where nasopharyngeal secretions cannot be obtained, an alternative specimen for rapid diagnosis has to be found. We have shown that, although the method has certain

Table 3. *Nasopharyngeal secretions examined for respiratory syncytial virus by fluorescent antibody technique, compared with cough/nasal swab preparations examined by the same method*

Diagnosis	Number examined	N.P.S.+		N.P.S.−	
		C/swab+	C/swab−	C/swab+	C/swab−
Bronchiolitis	17	14	1	—	2
Bronchitis	13	9	1	0	3*
Croup	2	1	—	—	1
U.R.T.I.	2	2	—	—	—
Pertussis	1	0	0	0	1†
Totals	35	26	2	0	7

* Parainfluenza virus type 3 isolated from two cases.

† Adenovirus type 5 isolated.

There was only one case in which both cough/nasal swab and nasopharyngeal secretion were positive by the F.A. technique but virus isolation failed (S. T., of Table 1).

Copositivity: 26/28 = 93 %; conegativity: 7/7 = 100 %; overall agreement: 33/35 = 94 %. After Buck & Gart (1966).

N.P.S. = nasopharyngeal swab; C/swab = cough/nasal swab; U.R.T.I. = upper respiratory tract infection; F.A. = fluorescent antibody.

Table 4. *Cough/nasal swab preparations examined for R.S.V. by fluorescent antibody technique compared with isolations*

Diagnosis	No. of C/N swabs examined	R.S.V. culture positive		R.S.V. culture negative	
		F.A. positive	F.A. negative	F.A. positive	F.A. negative
Pneumonia	5	4	0	0	1
Bronchitis	3	0	0	0	3
U.R.T.I.	6	3	0	0	3
Croup	2	0	1	0	1*
Totals	16	7	1	0	8

* Parainfluenza virus Type 1 isolated.

Copositivity: 7/8 = 88 %; conegativity: 8/8 = 100 %; overall agreement: 15/16 = 94 %. After Buck & Gart (1966).

R.S.V. = Respiratory syncytial virus. C/N = Cough/nasal. F. A. = Fluorescent antibody.

disadvantages, cough/nasal swab preparations can be used dependably for this purpose. Intact cells, which are essential for diagnosis, are very scanty in number, therefore much more time has to be spent in examining the stained specimens under the fluorescent microscope. In addition, reliable discrimination requires considerable experience because of the amount of non-specific fluorescence evident in these preparations. When examined by an experienced worker, however, a correct positive diagnosis can be made, as has been proved here by examining parallel nasopharyngeal secretions and cough/nasal swabs by fluorescent antibody technique and confirming positive results by the subsequent isolation of the virus. It must be emphasized again that the technique that we have devised and described in this paper is quite different from those previously used as a means of early detection of R.S. virus in throat swabs (Gray *et al.* 1968); the purpose of

swabbing the patient is to remove intact cells for examination by the fluorescent antibody technique.

Although it can also be found in less severe respiratory illnesses, R.S. virus causes severe infection in children mainly under the age of 2 years and is capable of causing bronchiolitis, pneumonia and death (Channock *et al.* 1961; Holzel *et al.* 1963; Elderkin, Gardner, Turk & White, 1965; Holzel *et al.* 1965; Gardner *et al.* 1967; Aherne *et al.* 1970). Even in this present small series of cases, 35 children mainly under the age of 1 year from whom both cough/nasal swabs and nasopharyngeal secretions were taken, and a group of 16 older children from whom only cough/nasal swabs were taken, R.S. virus was detected in 28 of the first series and eight of the second series (80 % and 50 % respectively). The total virus isolation rate was 89 % in the first series and 56 % in the second series. The failure rate for the detection of R.S. virus in cough/nasal swab preparations when compared with nasopharyngeal secretion was only two out of 28; the failure rate for cough/nasal swabs alone, as judged by virus isolation, was 1 out of 8. There was 1 patient (S. T., Table 1) in which both cough/nasal swab preparations and nasopharyngeal secretions were positive but no R.S. virus was isolated. This child had been ill for a fortnight before a specimen had been taken. Although specific R.S. virus fluorescence was found in both preparations, the fluorescence was duller and gave the appearance of perhaps being blocked by the child's own antibody production. We have observed on a number of occasions this sort of fluorescence in secretions taken from patients some days after their first positive specimen and have found difficulty in isolating the virus from the second specimen which exhibits this type of fluorescence (P. S. Gardner & J. McQuillin, unpublished results). These are all hospital studies and are, perhaps, biased in that only the child who is sufficiently ill with a respiratory infection to necessitate admission is examined.

The typical history of a bronchiolitis is of a severe illness which is usually preceded by a mild cold. It is therefore the young baby in the susceptible age group acquiring an upper respiratory infection who is in danger of developing a severe lower respiratory infection when R.S. virus is the infecting agent. It would be an advantage if these children could be investigated by a rapid diagnostic technique during the early stages of their upper respiratory tract infection when they are under the care of the general practitioner at home. It is well within the scope of the general practitioner to take a cough/nasal swab and send it to the virus laboratory for examination, provided facilities for transport of the specimen are available. Once we are able to diagnose R.S. virus infection at this early stage, it is possible that bronchiolitis might be prevented as antiviral agents become available. The success of the technique depends on the cough/nasal swabs being efficiently taken with much adhering secretion and cells; preparations containing too few cells could lead to false negative results.

Viruses other than R.S. virus are implicated in respiratory infections of older children and adults. It is known that influenza A can be seen in secretions of the respiratory tract using the fluorescent antibody technique (Hers *et al.* 1968), and it is possible that the cough/nasal swabs could become the method of choice in diagnosing infections in older children and adults.

Croup, principally caused by the parainfluenza group of viruses, is an infection of children between the ages 1 and 5 (Chanock *et al.* 1963). Techniques for the rapid diagnosis of parainfluenza virus infections are being developed at this Centre and perhaps could be applied to cough/nasal swab preparations (Gardner, 1969).

If cough/nasal swab preparations can be successfully used for the rapid diagnosis of these viruses, then the virus laboratory could carry out a diagnostic service for a much wider section of the population, should it be considered necessary to do so. This would be particularly important should antiviral agents become available.

The series of 35 children of the younger age group has shown, when isolation and fluorescent techniques are used together, that an 89% diagnostic rate can be achieved; 80% of these infections were due to R.S. virus, for which fluorescent techniques are available, and all of the children in this series were diagnosed on the day of admission to hospital. The second series of 16 children, from whom only cough/nasal swab preparations were taken, has shown that even in children over the age of 2 a high diagnostic rate can be obtained on the day of admission.

Should rapid diagnosis of parainfluenza, influenza A and adenovirus infections become possible in the future with cough/nasal swab preparations, then with relatively simple techniques, a diagnosis, within hours of onset of respiratory illness, could be confidently expected in between 80 and 90% of infants and in a high percentage of older children and adults with respiratory virus infection.

We wish to thank Professor S. D. M. Court, his clinical colleagues and their ward sisters and medical staff at all the hospitals for their collaboration. Special thanks are due to the Scientific and Research Sub-committee of the Royal Victoria Infirmary and to the Medical Research Council for grants which made this work possible.

REFERENCES

AHERNE, W., BIRD, T., COURT, S. D. M., GARDNER, P. S. & MCQUILLIN, J. (1970). Pathological changes in virus infections of the lower respiratory tract in children. *Journal of Clinical Pathology* **23**, 7.

BUCK, A. A. & GART, J. J. (1966). Comparison of a screening test and a reference test in epidemiologic studies. *American Journal of Epidemiology* **83**, 586.

CHANOCK, R. M., KIM, H. W., VARGOSKO, A. J., DELEVA, A., JOHNSON, K. M., CUMMING, C. & PARROTT, R. H. (1961). Respiratory syncytial virus. I. Virus recovery and other observations during 1960 outbreak of bronchiolitis, pneumonia and minor respiratory diseases in children. *Journal of the American Medical Association* **176**, 647.

CHANOCK, R. M., PARROTT, R. H., JOHNSON, K. M., KAPIKIAN, A. Z. & BELL, J. A. (1963). Myxoviruses: parainfluenza. *American Review of Respiratory Diseases* **88** (Suppl.), 152.

DOANE, F. W., ANDERSON, N., CHATYANONDA, K., BANNATYNE, R. M., MCLEAN, D. M. & RHODES, A. J. (1967). Rapid laboratory diagnosis of paramyxovirus infections by electron microscopy. *Lancet* ii, 751.

DOANE, F. W., ANDERSON, N., ZBITNEW, A. & RHODES, A. J. (1969). Application of electron microscopy to the diagnosis of virus infections. *Canadian Medical Association Journal* **100**, 1043.

ELDERKIN, F. M., GARDNER, P. S., TURK, D. C. & WHITE, A. C. (1965). Aetiology and management of bronchiolitis and pneumonia in childhood. *British Medical Journal* ii, 722.

GARDNER, P. S. (1969). Rapid diagnosis by fluorescent antibody techniques. Third International Symposium on Aerobiology.

GARDNER, P. S. & MCQUILLIN, J. (1968*a*). Application of immunofluorescent antibody technique in rapid diagnosis of respiratory syncytial virus infection. *British Medical Journal* iii, 340.

GARDNER, P. S. & MCQUILLIN, J. (1968*b*). Viral diagnosis by immunofluorescence. *Lancet* i, 597.

GARDNER, P. S., TURK, D. C., AHERNE, W. A., BIRD, T., HOLDAWAY, M. D. & COURT, S. D. M. (1967). Deaths associated with respiratory tract infection in childhood. *British Medical Journal* iv, 316.

GRAY, K. G., MACFARLANE, D. E. & SOMMERVILLE, R. G. (1968). Direct immunofluorescent identification of respiratory syncytial virus in throat swabs from children with respiratory illness. *Lancet* i, 446.

HAIRE, M. (1968). Rapid identification of rubella-virus antigen from throat swabs. *Lancet* i, 920.

HERS, J. F. Ph., VAN DER KUIP, L. & MASUREL, N. (1968). Rapid diagnosis of influenza. *Lancet* i, 510.

HOLZEL, A., PARKER, L., PATTERSON, W. H., CARTMEL, D., WHITE, L. L. R., PURDAY, R., THOMPSON, K. M. & TOBIN, J. O'H. (1965). Virus isolations from throats of children admitted to hospital with respiratory and other diseases in Manchester, 1962–64. *British Medical Journal* i, 614.

HOLZEL, A., PARKER, L., PATTERSON, W. H., WHITE, L. L. R., THOMPSON, K. M. & TOBIN, J. O'H. (1963). Isolation of respiratory syncytial virus from children with acute respiratory disease. *Lancet* i, 295.

JONCAS, J., BERTHIAUME, L., WILLIAMS, R., BEAUDRY, P. & PAVILANIS, V. (1969). Diagnosis of viral respiratory infections by electron microscopy. *Lancet* i, 956.

MCQUILLIN, J. & GARDNER, P. S. (1968). Rapid diagnosis of respiratory syncytial virus infection by the immunofluorescent antibody techniques. *British Medical Journal* i, 602.

MILLER, D. L. & TAYLOR, C. E. D. (1968). Evaluation of a new diagnostic test. *Lancet* i, 697.

STURDY, P. M., MCQUILLIN, J. & GARDNER, P. S. (1969). A comparative study of methods for the diagnosis of respiratory virus infections in childhood.

EXPLANATION OF PLATE

Fig. 1. Two cells in a cough/nasal swab preparation stained by the fluorescent antibody technique for R.S. virus. There is a considerable amount of non-specific fluorescence. Magnification: × 1900.

Fig. 2. One cell in a cough/nasal swab preparation showing good specific fluorescence when stained by the fluorescent antibody technique for R.S. virus. Considerable non-specific fluorescence still visible. Magnification: × 1900.

Fig. 3. Two cells in a nasopharyngeal secretion stained by the fluorescent antibody technique for R.S. virus. There is little specific background fluorescence. Magnification: × 1900.

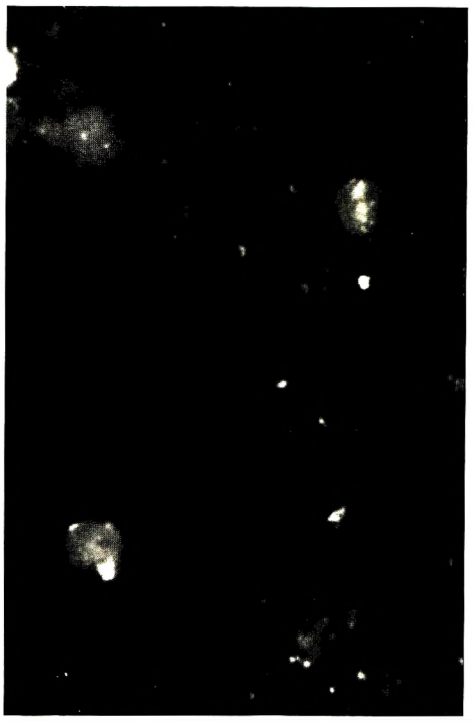

Fig. 1

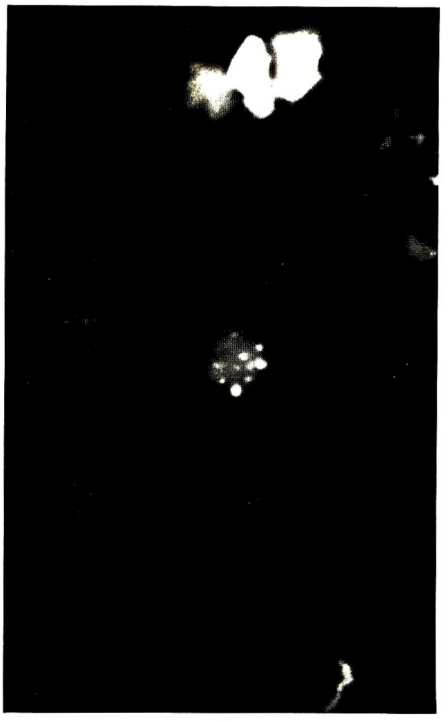

Fig. 2

Fig. 3

J. Hyg., Camb. (1970), **68**, 293
Printed in Great Britain

The use of gamma radiation for the elimination of *Salmonella* from frozen meat

By F. J. LEY, T. S. KENNEDY and K. KAWASHIMA*
Wantage Research Laboratory (AERE), Wantage, Berks.

and DIANE ROBERTS and BETTY C. HOBBS
Food Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W.9

SUMMARY

The use of a gamma radiation process for the elimination of *Salmonella* from frozen meat is considered with particular reference to the treatment of boned-out horsemeat and kangaroo meat imported into the UK and intended for use as pet meat.

Examination of dose/survival curves produced for several serotypes of *Salmonella* in frozen meat shows that a radiation dose of 0·6 Mrad. will reduce a population by at least a factor of 10^5. The influence on the radiation resistance of salmonellas of such factors as preirradiation growth in the meat and temperature during irradiation have been examined and considered. It is also demonstrated with both preinoculated and naturally contaminated meat that postirradiation storage in the frozen state does not lead to the revival of irradiated salmonellas.

The properties of *Salmonella* survivors deliberately produced in meat using conditions of irradiation designed to simulate a commercial process are studied after six recycling treatments through the process. There were no important changes in characteristics normally used for identification of *Salmonella* but radiation resistance was lowered. Survivors grown *in situ* in meat after irradiation showed an abnormally long lag phase, and removal of competitive microflora in meat by the radiation treatment can influence the growth of salmonellas.

INTRODUCTION

Meat and meat products including poultry are perhaps the commonest known vehicles of *Salmonella* infection. Pet meat sold raw has also caused concern as a potential source of human salmonellosis, contamination spreading to human foods from knacker meat (Beasley *et al.* 1967) or from imported meats such as boneless horsemeat (Galbraith *et al.* 1962; Hobbs, 1965) or kangaroo meat (Anderson, Crowder & Woodruff, 1964) which are used in this trade. The high rate of contamination of frozen carcasses or boneless horsemeat from three countries in South America is confirmed by the investigations of van Schothorst & Kampelmacher (1967) in the Netherlands where this meat is often included in minced meat for human consumption.

* Present address: Food Research Institute, Ministry of Agriculture and Forestry, Tokyo, Japan.

In an attempt to obviate the danger associated with the raw pet-meat trade in the UK, new regulations (The Meat (Sterilization) Regulations 1969) came into operation on 1 November 1969 superseding those made in 1960. These regulations require that knacker meat and meat imported other than for human consumption as well as butchers' meat or imported meat which in either case is unfit for human consumption, must now be sterilized before entering the chain of distribution. Sterilization is defined in terms of the processes to be applied. In relation to the pet meat trade, 'sterilized' means treated by boiling or steam under pressure until every piece of meat is cooked throughout. Where the trade is concerned, this process must present practical problems to importers of frozen meat in large blocks, in addition to eliminating an established market in raw pet-meat. Furthermore, the heat process, even if carried out effectively, must be supervised to ensure that recontamination of the cooked meat, which is a distinct possibility (Galbraith *et al.* 1962), does not occur.

In contrast to the heating process, gamma radiation will penetrate blocks of frozen meat and inactivate bacteria without changing the physical state of the meat. Frozen meat could be irradiated in its original package as imported, no handling of the meat is involved and cross-contamination between unprocessed and processed meat at a radiation plant is readily avoided. In describing the efficacy of a gamma-radiation process for the elimination of salmonellas from frozen boneless meat Ley (1962), while confident of the technical feasibility of such an operation, referred to the legal situation surrounding the use of the process as the main difficulty in implementation; since that time important steps have been taken to clarify the position.

Following recommendations published in the report from a Ministry of Health Working Party on Food Irradiation, 1964, regulations controlling the use of ionizing radiation for the treatment of food for *human* consumption were made and came into force in 1967. The regulations impose a system of prohibitive control similar to that already adopted for certain classes of food additives. Each specific process must be approved through the Ministry of Health Advisory Committee on the Irradiation of Food and a memorandum issued by the committee includes a broad description of the nature of the scientific evidence required. Microbiological evidence forms a part of this requirement and the studies presented in this paper are directed to the provision of data related to the control of salmonellas in frozen meats with particular emphasis on imported frozen boneless meat intended as pet meat. Legally, irradiation of such meat is not subject to the Food (Control of Irradiation) Regulations 1967, but such a process has many features in common with irradiation of meat for sale for human consumption. The scrutiny of scientific data related to its use is appropriate, not only with regard to the effectiveness, but also in the light of possible changes in the properties of salmonellas which might survive radiation treatment and in certain circumstances multiply.

This paper gives quantitative inactivation data following the preliminary studies reported by Ley, Freeman & Hobbs (1963). The data relate to the choice of radiation dose for the elimination of salmonellas from frozen horsemeat, kangaroo meat and veal. The influence on radiation resistance of preirradiation

growth conditions and of temperature during irradiation was investigated as well as the possibility of postirradiation recovery during storage. The radiation resistance and biochemical and serological properties of survivors of irradiation, deliberately produced under simulated practical conditions of the proposed process, were examined and their growth rate measured and compared with that of normal salmonellas.

MATERIALS AND METHODS

Meat

Imported frozen boneless raw horsemeat, kangaroo meat and veal. For experiments requiring meat naturally contaminated with salmonellas, samples were obtained from the remainder of those blocks used for routine examination at Colindale which were shown to be positive. These samples were stored in the frozen state until required.

Salmonella serotypes

Six serotypes were used during the course of these studies—*Salmonella typhimurium* phage type 14, *S. senftenberg* 1502, *S. good*, *S. oranienburg*, *S. anatum*, *S. minnesota*. These serotypes occur frequently in imported horsemeat. *S. typhimurium* was selected for detailed study because it is the predominant serotype isolated from humans (Vernon, 1969). The particular phage type 14 was used because it is streptomycin resistant and thus marked for easy recognition, also it is comparatively radiation resistant. *S. senftenberg* 1502 was chosen because of its high resistance to freezing, also *S. seftenberg* is commonly found in foods of animal origin.

Radiation source

The Spent Fuel Element Assembly was used as a source of gamma radiation for the treatment of naturally contaminated frozen meat used in the storage experiment; the dose rate was 1·0 Mrad./hr. In the other investigations a 2000 curie Cobalt-60 'hot spot' source with a dose rate of 0·8 Mrad./hr. was used. Both sources are described by Ley & Rogers (1968).

Pretreatment of meat by irradiation

In all experiments involving quantitative recovery of salmonellas, with the exception of growth-rate studies, the meat was irradiated at 1·0 Mrad. in the frozen state ($-15°$ C.) as a routine procedure before use. This treatment improves recovery by removing interfering microflora. The validity of the use of irradiated meat as substrate for subsequent experiments on the radiation resistance of salmonellas was investigated in preliminary studies; the growth rate, recovery and resistance of salmonellas were compared in irradiated and unirradiated meat.

Inoculation of meat

Stock salmonella cultures were maintained on Dorset egg medium, transferred for experimental work to nutrient agar slopes and incubated for 17 hr. at 37° C. Using M/15 phosphate buffer the growth was removed, washed twice, and then resuspended in the buffer. Appropriate volumes of buffer were mixed with meat exudate for inoculation. Horsemeat was thawed and cut into 20 g. samples which were then placed individually into sterile aluminium cans. Inoculations were made using an Agla syringe, 0·3 ml. being transferred half to the centre and half to the outside of the meat.

Preirradiation growth conditions

For those experiments in which the salmonellas were grown in the meat before irradiation, the buffer suspension was diluted with ¼ strength Ringer solution and then transferred to meat exudate. In order to obtain dose/survival curves covering more than 5 log. cycles it is necessary to have initial numbers of organisms before irradiation of at least 10^8/g. This number was obtained by inoculation of the meat samples with 10^2/g. followed by incubation for 17 hr. or 2 days at 37° C., 7 days at 22° C. or inoculation with 10^6/g. followed by 2 days incubation at 22° C. For experiments not involving preirradiation growth of salmonellas in the meat, an inoculum of *ca.* 10^8/g. was added directly to the samples.

Temperature during irradiation

Samples for irradiation at −15° C. were frozen rapidly to this temperature in the cold room (48 min.) and irradiated in a vacuum flask containing a freezing mixture of ammonium chloride and powdered ice (−15° C.). Thermocouple measurements showed a variation from the inside to the outside of the meat of ±2° C. during the longest period of irradiation. Crushed ice was used in a similar way for meat irradiated at 0° C. Samples were also irradiated at room temperature which was *ca.* 20° C.

Influence of postirradiation storage on recovery

Recovery was examined quantitatively using artificially contaminated meat and qualitatively using naturally contaminated meat.

(*a*) *Artificially contaminated meat.* Twenty-four 20 g. meat samples were used each with counts of *ca.* 10^8/g. of *S. typhimurium*, this number was attained by inoculation with 10^2/g. followed by incubation at 37° C. for 17 hr. After freezing to −15° C., 12 samples were irradiated at 0·5 Mrad. calculated to give 10^3/g. survivors and 12 were kept as unirradiated controls. Viable counts were made at intervals over a 10 week storage period at −15° C. using solid media (agar).

(*b*) *Naturally contaminated meat.* One-hundred 50 g. samples (controls) were taken at random from various blocks of frozen meat. Each sample was placed individually in a polythene bag and heat sealed. These samples were returned to frozen store while the remainder of the meat blocks were packed lightly together in insulated containers for irradiation in the frozen state. The meat was packed

in a manner which gave a dose distribution through the meat within the range 0·5–0·75 Mrad. After irradiation, a further 100×50 g. samples were taken. Half the controls and half the samples of irradiated meat were examined after 6 days' storage ($-15°$ C.) and the remainder after 10 weeks. In addition, 12×10 g. samples were taken for total counts, six from the meat prior to irradiation and six immediately after irradiation.

Performance of viable counts, both total and of salmonellas, is described in a later section. The qualitative examination for salmonellas of the 50 g. samples referred to in (*b*) was carried out as follows: each sample was thawed and finely chopped and 25 g. added to 25 ml. $\frac{1}{4}$ strength Ringer solution and this mixture added to 50 ml. double strength Selenite F (Liefson, 1936), modified by replacement of lactose with mannitol and sterilization by Seitz filtration. A further 25 g. was added to tetrathionate enrichment broth (medium A of Rolfe, 1946) in a similar manner. Incubation was for 72 hr. at 37° C. Deoxycholate-citrate lactose agar (Hynes, 1942), modified by the addition of 1 % sucrose, and Oxoid bismuth sulphite agar were inoculated from the tubes of enrichment media and incubated for 48 hr. at 37° C. Characteristic colonies from the plates were examined and identified as *Salmonella* using biochemical and serological tests.

Production and isolation of radiation survivors

Meat samples were inoculated with 10^2/g. and incubated for 17 hr. at 37° C. to give a count of *ca.* 10^8/g. After freezing at $-15°$ C., the samples were irradiated at 0·65 Mrad. to reduce the count to 10^2/g. Surviving organisms were recovered directly on Oxoid nutrient agar and also through Selenite F and tetrathionate broth on deoxycholate citrate sucrose agar; they were transferred to Dorset egg media for storage. This procedure is referred to as 1 cycle of treatment. In the case of *S. typhimurium* up to 6 cycles of treatment were used.

Dose/survival curves

The fraction of surviving organisms was calculated using the average count from two unirradiated control samples, one plated at the start and the other at the end of the longest irradiation period. Individual samples of meat were used to obtain the data for each of the 6–8 points used for the construction of each dose/survival curve.

Three dose/survival curves were prepared on separate occasions for each treatment in the studies, using *S. typhimurium*, of the influence on resistance of pre-irradiation growth conditions, of temperature during irradiation, and of different meat substrates, and in studies on the resistance of radiation survivors. A common regression line was fitted to these curves by means of the method of least squares. The regression line is in the form $y = ax + b$, where y is the logarithm of the surviving fraction, x is the dose of radiation, a is the slope of the line and b is the logarithm of the extrapolation number (Alper, Gillies & Elkind, 1960). For comparative purposes D 10 values (dose required to reduce the number of survivors to one-tenth) were calculated from the linear part of the curves and confidence limits derived following an analysis of variance. In the studies illustrating the shapes of

curves obtained with different serotypes, one dose/survival curve was produced in each case using solid media and at least two curves using liquid media.

Biochemical and serological properties of surviving organisms

Phage typing, serological typing, fermentation and other cultural studies were carried out on the strain of *S. typhimurium* used in these studies before irradiation and after each of six cycles of radiation treatment. Serological patterns only of control cultures of *S. senftenberg*, *S. good*, *S. oranienburg*, *S. anatum* and *S. minnesota* and those of each strain after one cycle of treatment were examined.

Growth rate

The growth rate of salmonellas in meat was studied in the following situations, (i) as natural contaminants or after inoculation into untreated meat, (ii) after inoculation into irradiated meat (0·65 Mrad.), (iii) after inoculation of first cycle survivors into irradiated meat (0·65 Mrad.), (iv) survivors *in situ* in meat (previously artificially contaminated with high numbers of salmonellas) following irradiation with 0·65 Mrad.

(i) Horsemeat suspected to be naturally contaminated with salmonellas was finely chopped, well mixed and distributed in 12 g. quantities into sterile screw cap bottles. The 0 hr. sample was examined immediately and the remainder incubated at 30° C. for different periods up to 48 hr., an individual sample being used for each time interval—four growth curves were produced on separate occasions. Salmonella counts were performed using liquid media by the Most Probable Number (MPN) technique (see following section). Similarly curves were constructed showing growth in normal horsemeat following artificial inoculation with *S. typhimurium* or *S. anatum* at different concentrations; 1 ml. of an appropriate dilution of a 6 hr. broth culture was added to each 12 g. sample.

(ii) and (iii) Meat was irradiated at − 15° C. with a dose of 0·65 Mrad. and after thawing inoculated with either normal (untreated) *S. typhimurium* or first cycle survivors of this serotype. Growth curves in the meat were obtained following incubation and counting as in (i).

(iv) 1 ml. of a suspension of *S. typhimurium* in meat exudate (10^{10}/ml.) was inoculated into each of 7 × 12 g. samples of horsemeat and a further 7 samples inoculated with 1 ml. of a 1 in 100 dilution of the suspension, thus giving samples containing two concentrations of survivors for separate growth experiments. After thawing growth curves were produced following incubation as in (i) but using a solid medium (agar). When liquid media were used in this situation as enrichment or pre-enrichment media, and counts determined by the MPN method, the phenomenon of 'skipping' was observed (North, 1961) invalidating the counting technique; this is the subject of a further investigation.

Performance of viable counts

Salmonella counts using solid media (agar). Each meat sample, after being thawed (when applicable) at room temperature for 30 min., was macerated for 1 min. with 100 ml. sterile distilled water in an MSE top drive macerator. 1 ml. portions were

used for plate counts; when necessary the macerated meat suspension was diluted with $\frac{1}{4}$ strength Ringer solution. The surface plating technique used for high numbers of survivors was a modified form of the method of Miles & Misra (1938); poured plates were used when low numbers only were expected to survive.

Deoxycholate citrate sucrose agar, 'Oxoid' MacConkey agar, no. 2, 'Oxoid' Salmonella Shigella agar, 'Oxoid' triple sugar iron agar (TSI) and 'Oxoid' brilliant green agar (BGA) were tested and compared with 'Oxoid' nutrient agar (NA) for recovery of salmonellas from meat following irradiation up to 0·5 Mrad., the highest dose used; NA was found to be satisfactory by Ley *et al.* (1963) when meat used in experiments had been irradiated and the requirement was merely to count salmonellas in the absence of other organisms. BGA gave the highest viable count of the four first mentioned media and gave the same results as those obtained using NA. Both media were used in many of the experiments reported; the results for BGA only are presented in these experiments.

The characteristic pink colour of *Salmonella* colonies on BGA was not evident below the surface of the agar in poured plates. To establish whether it could be assumed that both deep and surface colonies were all *Salmonella*, colonies taken at random were transferred to TSI agar from the BGA plates. When the meat samples used had been treated by irradiation before inoculation, few organisms other than *Salmonella* were to be expected to grow and all colonies transferred to TSI agar gave H_2S production and sugar fermentation reactions for *Salmonella* sp.; the square root of the number of colonies were taken at random for slide agglutinations and all gave positive reactions against *Salmonella* O and H antisera. On the basis of these tests all colonies were assumed to be salmonellas. When the requirement was to count salmonellas in a mixed flora, for example when unirradiated meat was used for inoculation, the use of BGA medium for counting was always accompanied by the above procedure for the detection of salmonellas.

Salmonella counts using liquid media. Each meat sample was macerated as described above but with 120 ml. $\frac{1}{4}$ strength Ringer solution. The MPN technique based on the probability tables of McCrady (1918) was used for salmonella counts. For the dose/survival curve experiments in which the requirement was to count very low numbers of salmonellas, lactose broth pre-enrichment medium was used according to North (1961). Selinite F enrichment medium was used elsewhere with incubation at 37° C. for up to 48 hr. All enrichment media were inoculated on both 'Oxoid' bismuth sulphite agar and deoxycholate citrate sucrose agar for colony isolation. Identification of isolated colonies as *Salmonella* was confirmed by biochemical and serological tests.

Total viable counts and coliforms. These were performed by the technique of Miles & Misra (1938) using blood agar plates incubated aerobically and anaerobically and MacConkey agar incubated aerobically at 37° C. for 48 hr.

RESULTS

The preliminary studies showed the suitability of meat which had been previously irradiated for use in dose/survival curve experiments. *Salmonella* grew in

the meat satisfactorily and the growth curves were of expected shape (Fig. 1). Following inoculation, more salmonellas were recovered with less variation between samples with irradiated meat ($1{\cdot}9 \times 10^8$/g. with S.E. $\pm 0{\cdot}22$) compared with unirradiated meat ($7{\cdot}7 \times 10^7$/g. $\pm 1{\cdot}95$). Furthermore, a dose/survival curve (irradiation at $-15°$ C.), prepared in previously irradiated meat using *S. typhimurium* was similar in shape to that obtained in the unirradiated meat and the D10 values

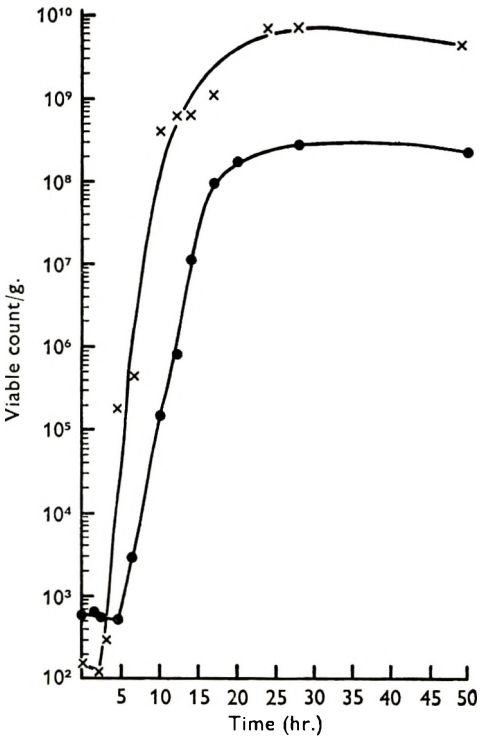

Fig. 1. Rate of growth of *S. typhimurium* at 30° C. (●) and 37° C. (×) following inoculation into irradiated horsemeat (1·0 Mrad.).

Table 1. *The influence of preirradiation growth conditions in horsemeat on the radiation resistance of* S. typhimurium, *results given as D*10 *values (krad.)*

Preirradiation growth conditions following inoculation	Temperature during irradiation (°C.)	
	−15	+20
No growth**	86·1 (80·7–92·4)	48·7 (45·3–52·7)
22° C. for 2 days	*	51·1 (45·1–58·9)
22° C. for 7 days	*	86·3 (76·4–99·1)
37° C. for 17 hr.	92·6 (87·9–97·7)	62·7 (54·9–73·0)

95 % Confidence limits given in parentheses.
* Not tested. ** Organisms merely added to meat.

were also similar, i.e. 92·6 krad. (87·9–97·7) for the former and 103·7 krad. (93·3–116·8) for the latter. It can be concluded that the previous irradiation of the meat does not produce any toxic effect which influences the growth, recovery or radiation resistance of *Salmonella* subsequently inoculated into the meat.

Table 2. *The D*10 *values (krad.) for* S. typhimurium *in various meats irradiated at different temperatures following inoculation and growth at* 37° *C. for* 17 *hr.*

Temperature during irradiation (°C.)	Horsemeat	Kangaroo meat	Veal
−15	92·6 (87·9–97·7)	92·8 (86·9–99·6)	96·3 (91·2–102·0)
+20	62·7 (54·9–73·0)	55·5 (50·5–61·8)	55·8 (51·0–61·6)

95 % Confidence limits in parentheses.

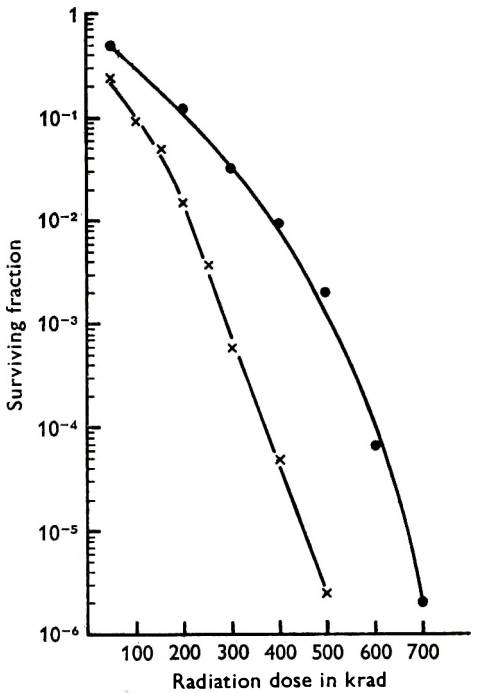

Fig. 2. Dose/survival curves for *S. typhimurium* irradiated in horsemeat at −15° C. (●) Following inoculation and growth in the meat at 37° C. for 2 days; (×) following inoculation but without preirradiation growth in the meat.

The D10 values quoted in Tables 1 and 2 were read from dose/survival curves which were linear over the dose range studied, i.e. up to 0·6 Mrad. for frozen meat and 0·4 Mrad. for unfrozen meat and covering between 5 and 6 log. cycles of inactivation in each case. The results in Table 1 show that preirradiation growth of *S. typhimurium* in meat under certain incubation conditions can have a

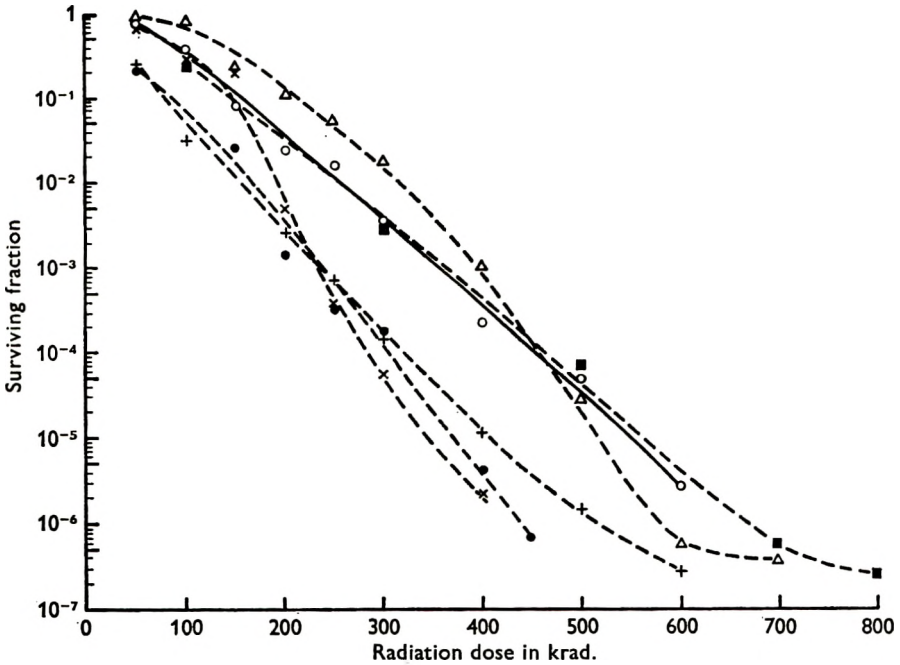

Fig. 3. Dose/survival curves for various serotypes of salmonellas in horsemeat prepared using solid recovery media. (Irradiation at −15° C. following preirradiation growth in the meat at 37° C. for 17 hr.) ×, *S. minnesota*; ●, *S. good*; +, *S. oranienburg*; ○, *S. typhimurium*; △, *S. senftenberg*; ■, *S. anatum*.

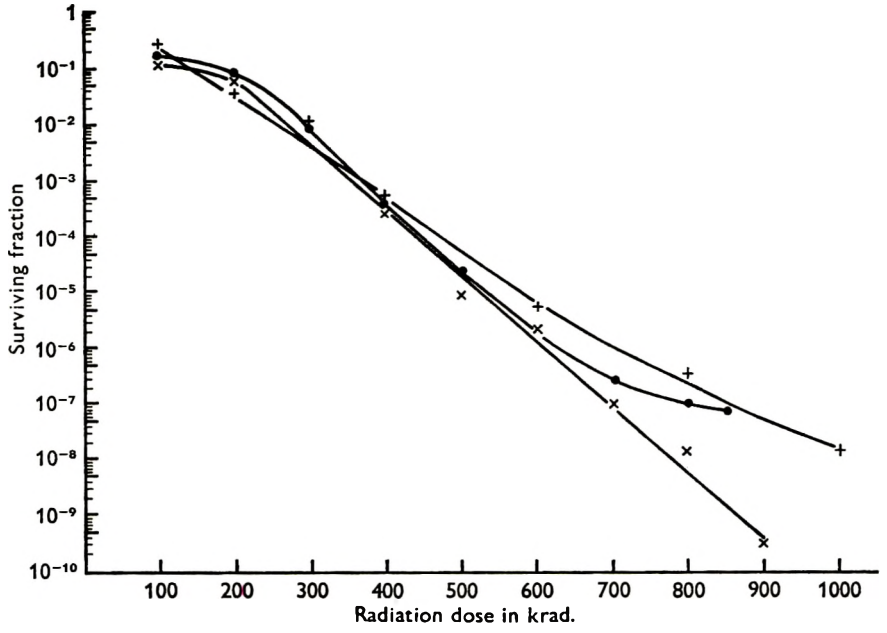

Fig. 4. Dose/survival curves for various serotypes of salmonellas in horsemeat prepared using the lactose broth pre-enrichment method (North, 1961). (Irradiation at −15° C. following preirradiation growth in the meat at 37° C. for 17 hr.) ×, *S. typhimurium*; ●, *S. senftenberg*; +, *S. anatum*.

significant effect on resistance. This is apparent with 7 days at 22° C. ($P < 0{\cdot}001$) and 17 hr. at 37° C. ($P < 0{\cdot}002$) when followed by irradiation at 20° C. compared with organisms merely added to the meat. With irradiation in the frozen state increase in resistance was also observed with 2 days at 37° C. as illustrated in Fig. 2; the non-linearity of the curve in this instance precludes the calculation of a D 10 value. After perusal of all the results in the light of the commercial process envisaged for frozen meat, preirradiation growth for 17 hr. at 37° C. was chosen as a standard procedure in subsequent experiments; longer incubation in practice leads to unacceptable deterioration of meat quality. Results in Table 2 show that there is no significant difference in the radiation resistance of *S. typhimurium* when different meats are used as substrates whether irradiated frozen or unfrozen ($P > 0{\cdot}5$ throughout). The marked increase in resistance due to freezing is evident

Table 3. *The effect of storage at* − 15° *C. on the surviving fraction of* S. typhimurium *in frozen horsemeat following irradiation with* 0·5 *Mrad.*

Storage (weeks)	Counts/g. Unirradiated	Counts/g. Irradiated	Surviving fraction
0	$1{\cdot}9 \times 10^8$*	$2{\cdot}7 \times 10^3$*	$1{\cdot}6 \times 10^{-5}$
1	$1{\cdot}6 \times 10^8$	$3{\cdot}0 \times 10^3$	$1{\cdot}8 \times 10^{-5}$
2	$2{\cdot}4 \times 10^8$	$3{\cdot}7 \times 10^3$	$1{\cdot}6 \times 10^{-5}$
3	$1{\cdot}1 \times 10^8$	$6{\cdot}2 \times 10^2$	$5{\cdot}7 \times 10^{-6}$
4	$1{\cdot}3 \times 10^8$	$6{\cdot}0 \times 10^2$	$4{\cdot}6 \times 10^{-6}$
5	$1{\cdot}2 \times 10^8$	$4{\cdot}7 \times 10^2$	$3{\cdot}8 \times 10^{-6}$
10	$6{\cdot}4 \times 10^7$	$1{\cdot}0 \times 10^2$	$1{\cdot}6 \times 10^{-6}$

* Mean of duplicate counts obtained on six individual meat samples; all other results are the means of duplicate counts on one sample.

with each type of meat; in each case the DMF (dose modifying factor = ratio of D 10 value at − 15° C. to D 10 value at 20° C.) is *ca.* 1·7. Using horsemeat only, irradiation at 0° C. was also studied and a D 10 value of 55·3 krad. (48·3–64·6) obtained which is not significantly different from that at 20° C. ($P > 0{\cdot}1$).

Curves depicted in Fig. 3 were obtained using solid media and those in Fig. 4 using liquid media. In all these experiments attempts were made to obtain curves extending as far as is practicable. The curves are of various shapes with evidence of both shoulders and tails, the latter being particularly obvious with *S. senftenberg* and *S. anatum*. Although the shapes are similar with both media, the increased sensitivity of the liquid recovery technique results in curves passing through a greater number of log. cycles of inactivation, that for *S. typhimurium* covering more than nine log. cycles. The tails to the curves appear almost one log. cycle lower with the liquid media compared with solid media implying that tailing might be associated with limitations on estimating very low numbers of survivors. It can be concluded by inspection of all the curves presented that a reduction in numbers by at least a factor of 10^5 can be achieved in an initial population of any of the serotypes examined with a dose of 0·6 Mrad.

The effect of postirradiation storage at − 15° C. on the recovery of *S.*

typhimurium in frozen horsemeat is shown in Table 3. The dose of 0·5 Mrad. was chosen to give a convenient number of survivors. The variance between counts obtained for the unirradiated organisms over the whole storage period was not statistically different ($P > 0{\cdot}1$) from that obtained for the survivors of irradiation. Although the numbers of both the unirradiated organisms and irradiation survivors fall with time, it will be seen that when the number of survivors is expressed as a fraction of the unirradiated organisms, this fraction also decreases slowly with time

Table 4*a*. *Detection of* Salmonella *in naturally contaminated meat before and after irradiation at* $-15°$ *C.*

Dose (Mrad.)	Storage after irradiation	No. of samples examined	No. in which *Salmonella* detected	No. showing growth on selective media*		
				++	+	−
0	6 days	50	22	50	0	0
0·5–0·75	6 days	50	0	0	7	43
0	10 weeks	50	10	46	1	3
0·5–0·75	10 weeks	47	0	0	7	40

* On bismuth sulphite or deoxycholate citrate sucrose agar following enrichment broths. ++ = Heavy; + = light; − = no growth.

Table 4*b*. *Total viable counts on meat before and after irradiation (0·5–0·75 Mrad.) at* $-15°$ *C.*

	Sample no.	Orgs/g. Aerobic	Orgs/g. Anaerobic	Coliform bacilli
Before	1	500	500	< 500
	2	**	2×10^5	5×10^4
	3	2×10^5	3×10^4	1×10^4
	4	1×10^5	$2{\cdot}5 \times 10^5$	$2{\cdot}5 \times 10^5$
	5	2×10^5	2×10^5	2×10^5
	6	$1{\cdot}5 \times 10^5$	$1{\cdot}5 \times 10^5$	$1{\cdot}5 \times 10^5$
After	1–6	< 500 Throughout		Not found

** Unreadable.

Table 5. *The influence of the recycling* of survivors of irradiation* (S. typhimurium) *on their subsequent radiation resistance in horsemeat at* $-15°$ *C.*

Treatment	D10 value (krad.)
Uncycled (control)	92·6 (87·9–97·7)
First cycle survivors	67·5 (63·1–72·6)
Sixth cycle survivors	67·9 (60·1–77·9)
First cycle survivors (freezing only)	87·0 (80·5–94·7)

95% Confidence limits given in parentheses.

* Each cycle is as follows: inoculation of meat with 10^2/g. incubation for 17 hr. at 37° C. to give 10^8/g., freeze to $-15°$ C., irradiation with 0·65 Mrad., recover on nutrient agar.

Table 6. *The growth at* 30° *C. of* Salmonella *in naturally contaminated horsemeat or after inoculation with* S. anatum

Time (hr.)	MPN count/g. naturally contaminated Expt. no. 1	2	3	4	Range of total plate count/g. Aerobic	Anaerobic	MPN count/g. artificially inoculated Expt. no. 1	2	3	4	5	Range of total plate count/g. Aerobic	Anaerobic
0	0·5	3·0	4·0	5·0	10^5–10^6	10^5–10^6	0·3	0·5	5	70	130*	10^3–10^6	10^3–10^4
15–17	0·1	0·1	0·6	0·2	10^8–10^9	10^8–10^9	2	20	65	$> 1{\cdot}8 \times 10^6$	$> 1{\cdot}8 \times 10^6$	10^8–10^{10}	10^8–10^9
24	1	4	9	14	10^8–10^{10}	10^8–10^{10}	140	60	$5{\cdot}5 \times 10^2$	$> 1{\cdot}8 \times 10^6$	$> 1{\cdot}8 \times 10^6$	10^9–10^{10}	10^8–10^{10}
40	20	0	70	2·0	10^9–10^{10}	10^9–10^{10}	$> 1{\cdot}8 \times 10^4$	50	2×10^2	2×10^5	$> 1{\cdot}8 \times 10^8$	10^{10}	10^9–10^{10}
48	200	0	$2{\cdot}5 \times 10^3$	130	10^{10}–10^{11}	10^9–10^{10}	$2{\cdot}7 \times 10^4$	$2{\cdot}5 \times 10^3$	4×10^2	$> 1{\cdot}6 \times 10^8$	$> 1{\cdot}8 \times 10^8$	10^{10}	10^9–10^{10}

* *S. typhimurium.*

giving a one log. cycle fall at the tenth week. The number of samples in which salmonellas were detected in meat suspected of being naturally contaminated was reduced in storage (Table 4*a*) but the effectiveness of the radiation treatment is apparent. The effect on other bacterial species is also clear as shown by the extent of growth on selective media of mixed coliforms and by the fall in total viable count shown in Table 4*b*. Coliforms are not expected to survive the irradiation and the light growth observed in some of the irradiated samples are probably due to postirradiation contamination; a few polythene bags were found to be split.

Table 7. *Growth at* 30° *C. of* S. typhimurium *following inoculation into irradiated* (0·65 *Mrad.*) *horsemeat*

Time (hr.)	Salmonella MPN count*/g.	Total plate count/g.		Salmonella MPN count*/g.	Total plate count/g.	
		Aerobic	Anaerobic		Aerobic	Anaerobic
0	2×10^2	8×10^2	$4{\cdot}7 \times 10^2$	0·2	10	5
6	$2{\cdot}6 \times 10^3$	$2{\cdot}4 \times 10^3$	$1{\cdot}6 \times 10^3$	13	25	45
12	$6{\cdot}6 \times 10^6$	$4{\cdot}4 \times 10^7$	$4{\cdot}3 \times 10^7$	$6{\cdot}0 \times 10^4$	$5{\cdot}9 \times 10^4$	$5{\cdot}9 \times 10^4$
18	$1{\cdot}6 \times 10^8$	$7{\cdot}5 \times 10^7$	$8{\cdot}2 \times 10^7$	$6{\cdot}0 \times 10^6$	$7{\cdot}6 \times 10^6$	$7{\cdot}2 \times 10^6$
24	$2{\cdot}2 \times 10^9$	$3{\cdot}8 \times 10^9$	$3{\cdot}3 \times 10^9$	$1{\cdot}7 \times 10^8$	$2{\cdot}8 \times 10^8$	$3{\cdot}1 \times 10^8$
36	$1{\cdot}9 \times 10^9$	$1{\cdot}2 \times 10^{10}$	$1{\cdot}2 \times 10^{10}$	$2{\cdot}2 \times 10^9$	$1{\cdot}4 \times 10^{10}$	$1{\cdot}6 \times 10^{10}$
48	$4{\cdot}2 \times 10^8$	$1{\cdot}7 \times 10^{10}$	$1{\cdot}7 \times 10^{10}$	$3{\cdot}5 \times 10^9$	$1{\cdot}3 \times 10^{10}$	$1{\cdot}5 \times 10^{10}$

* Using Selenite F.

The radiation resistance of salmonellas which have survived freezing and irradiation showed a significant decrease ($P < 0{\cdot}001$) after 1 cycle of treatment and no further decrease after 6 cycles (Table 5); this effect is not accounted for by freezing alone. The serological pattern, 1, 4, 5, 12:i:1, 2 and phage type 14, of *S. typhimurium* remained unaltered throughout the six cycles but there was a slight change in the fermentation pattern. After the first cycle inositol fermentation appeared to have been induced and, after the second cycle, rhamnose fermentation; these reactions were held throughout the subsequent cycles. There were other slight variations in the fermentation pattern, e.g. differences in the length of time required for certain fermentations to become apparent, but these were probably of minor significance. The qualities which remained constant were the cultural characteristics and the serological reactions, and acriflavine reactions which suggested that the strain had not become degraded by the radiation treatment. The serological patterns of the other serotypes examined after one cycle of treatment showed no apparent changes.

The growth rate of normal salmonellas in untreated horsemeat is influenced by the initial numbers present; low numbers had difficulty in growing, whereas an inoculum of about 10^2/g. grew rapidly in competition with the natural flora (Table 6). In fact, the growth rate of the heavier salmonella inoculum was comparable with that achieved in the absence of other flora as shown in the results from the preliminary work given in Fig. 1. Even a low inoculum was able to grow well in meat which had been irradiated at 0·65 Mrad. in which the natural flora was reduced considerably in number, and the growth rate of first cycle survivors

of irradiation under the same conditions was very comparable (Tables 7, 8). In contrast, survivors of irradiation produced *in situ* in meat followed by incubation in the same meat showed a longer lag period whether the number of survivors was high or low (Table 9).

Table 8. *Growth at* 30° *C. of* S. typhimurium *survivors of first irradiation cycle following inoculation into irradiated* (0·65 *Mrad.*) *horsemeat*

Time (hr.)	Salmonella MPN count*/g.	Total plate count/g.	
		Aerobic	Anaerobic
0	3·5	10	5
6	12·0	5	15
12	$1·6 \times 10^3$	$8·8 \times 10^3$	$2·9 \times 10^3$
18	$1·3 \times 10^5$	$2·4 \times 10^5$	$2·1 \times 10^5$
24	$1·8 \times 10^8$	$2·1 \times 10^8$	$2·4 \times 10^8$
36	$3·3 \times 10^7$	$3·4 \times 10^9$	$3·9 \times 10^9$
48	$4·2 \times 10^8$	$2·6 \times 10^9$	$2·6 \times 10^9$

* Using Selenite F.

Table 9. *Growth at* 30° *C. of* S. typhimurium *survivors* in situ *in horsemeat following irradiation with* 0·65 *Mrad.*

Time (hr.)	Salmonella count*/g.	Total plate count/g.		Salmonella count*/g.	Total plate count/g.	
		Aerobic	Anaerobic		Aerobic	Anaerobic
0	$6·2 \times 10^3$	$2·7 \times 10^4$	$8·6 \times 10^3$	50	$8·4 \times 10^2$	$1·1 \times 10^2$
6	$2·0 \times 10^3$	$6·2 \times 10^3$	$4·6 \times 10^3$	15	$3·4 \times 10^2$	$3·1 \times 10^2$
12	$2·3 \times 10^3$	$5·0 \times 10^3$	$4·3 \times 10^3$	25	$2·3 \times 10^4$	$1·9 \times 10^4$
18	$5·1 \times 10^3$	$6·3 \times 10^3$	$5·5 \times 10^3$	$3·4 \times 10^4$	$6·2 \times 10^5$	$4·5 \times 10^5$
24	$3·5 \times 10^5$	$3·7 \times 10^5$	$3·8 \times 10^4$	$6·5 \times 10^6$	$1·7 \times 10^7$	u/c
36	$3·8 \times 10^6$	$6·3 \times 10^6$	$6·3 \times 10^6$	$6·9 \times 10^7$	$7·2 \times 10^7$	$6·5 \times 10^7$
48	$3·7 \times 10^7$	$6·6 \times 10^7$	$3·5 \times 10^7$	$1·1 \times 10^8$	$1·2 \times 10^8$	$1·3 \times 10^8$

* Using solid media.

DISCUSSION

The immediate penetrating property of gamma radiation combined with the precision with which required doses can be given, allows quantitative microbiological inactivation data to be produced in the laboratory under conditions very similar to those expected in a commercial operation. This is important since many factors of environment before, during and after irradiation can influence radiation resistance (reviewed by Bridges, 1964). The importance of using the particular food itself as substrate in the measurement of the radiation resistance of *Salmonella* was illustrated by Ley *et al.* (1963) who observed considerable differences in different foods which in turn all showed protective effects when compared with buffer as suspending medium. In view of the possibility that salmonellas in meat might have the opportunity to grow during slaughterhouse handling, meat was inoculated with a small number of organisms and given a period of growth before

freezing to the temperature (−15° C.) at which the meat is normally shipped. Preirradiation growth conditions in food are known to influence radiation resistance as observed in our investigations on salmonellas in corned beef (Ley, 1966). High resistance in vegetative bacteria is expected in the stationary phase of growth (Stapleton, 1955) and this was confirmed for salmonellas grown in nutrient broth by Licciardello, Nickerson & Goldblith (1968) who also showed that preirradiation growth temperature can influence resistance; the highest resistance was obtained at 37° C. This was the temperature used in our main experiments and held for 17 hr. after which time the organisms were at the beginning of the stationary phase of growth.

The D10 value obtained for *S. typhimurium* in frozen horsemeat is somewhat lower than that reported by Ley *et al.* (1963) but in this earlier work the meat was irradiated in solid CO_2. However, the striking increase in radiation resistance in meat in the frozen state compared with the unfrozen state confirms their findings with buffer suspensions. Since the commercial radiation process is expected to be applied at import to incoming frozen meat which, after treatment, is distributed in the frozen state, it would be unpractical to irradiate in the thawed state to take advantage of the lower dose requirement; besides, such a proposal would present an opportunity for mishandling with the possibility of microbial growth. Whilst freezing has a protective effect on salmonellas and other vegetative bacteria (Matsuyama, Thornley & Ingram, 1964*a*), it seems to have little effect on the resistance of bacterial spores (Matsuyama, Thornley & Ingram, 1964*b*). However, the data presented by Grecz (1965) indicate some increased sensitivity for *Clostridium botulinum* spores irradiated in buffer at −20° C. compared with ambient temperatures when the dose given was 0·7 Mrad.; such conditions are similar to those expected in the commercial process described in this paper. As regards the effect of irradiation on the quality of the meat itself, there is no disadvantage in the use of the higher dose in the frozen state since freezing protects against damage (Coleby *et al.* 1961; Harlan & Kauffman, 1965). The nature and reactivity of free radicals formed during irradiation is thought to account largely for this difference between the frozen and unfrozen state.

The dose/survival curves presented for different serotypes, though complex in shape, extend far enough to read off directly the dose of radiation required to reduce a population by a factor of at least 10^5. A dose of 0·6 Mrad. is suggested as the minimum to use in practice in a commercial process. In the irradiation of blocks of frozen meat in cartons of dimensions $20 \times 20 \times 10$ in. ($50 \times 50 \times 25$ cm.) which are ideal for handling and conveying, attenuation of the dose is expected. If the minimum dose at the centre is 0·6 Mrad., then a dose of 0·85 Mrad. is received at the outside of the block resulting in inactivation factors for *Salmonella* varying between 10^5 and 10^8. This level of inactivation seems adequate considering that low numbers of salmonellas are expected in frozen meat; 5 viable organisms/gram was the highest number recorded during these studies. Mossel, van Schothorst & Kampelmacher (1968) showed that 0·6 Mrad. was adequate for the treatment of frozen poultry when the absence of Enterobacteriaceae flora in drip fluid was used as a measure of effectiveness. The same authors also reported the efficacy of this

dose for frozen red meats using the same criteria. Our own experiments comparing horsemeat, kangaroo meat and veal indicate no differences in radiation resistance for salmonellas between these meats as substrates, although salmonellas in crab-meat, studied by Dyer, Anderson & Dutiyabodhi (1966), are more radiation resistant.

It is only a remote possibility that salmonellas surviving irradiation, expected to be $< 1/10^5$ g., would have the opportunity to grow and recontaminate meat earmarked for radiation treatment, particularly if precautions are taken at a radiation plant to separate incoming from outgoing material, and to keep the conveyor system clean. However, it is encouraging to note that *S. typhimurium* recycled through the process shows reduced radiation resistance. Other work has shown, on the one hand, no change in resistance with *S. gallinarum* after 14 consecutive cycles of radiation treatment (Erdman, Thatcher & MacQueen, 1961*a*), and on the other, increased resistance with the several serotypes examined after at least eight cycles, though not with *S. typhimurium* unless followed by storage (Idziak & Incze, 1968) and after at least six cycles (Licciardello *et al.* 1969). However, the authors of the first two papers, referring to the characteristics of recycled *Salmonella* species, note no change in the reactions normally used in identification. Apart from two minor changes in sugar fermentation, our own results support the same conclusion and it is unlikely therefore that any practical problems would arise in this connexion.

Repair mechanisms have been shown to operate in bacteria following ultraviolet irradiation (reviewed by Witkin, 1969) but the cells of *Salmonella* inactivated in meat by gamma irradiation do not regain viability during frozen storage; it appears rather that the surviving organisms are reduced in number. However, survivors could have the opportunity to grow when the meat is thawed before use or allowed to thaw at some stage in distribution through mishandling. It is an advantage that survivors in the meat exhibit a longer lag phase than unirradiated salmonellas but it is important to note that irradiation used as envisaged in this application, which is similar in aim to a heat pasteurization process, causes a considerable reduction in the total microflora thus providing a medium subsequent to radiation treatment, which would be less inhibitory to the growth of salmonellas than unirradiated meat. Our results showing the influence of competing flora on the growth of *Salmonella* support the findings of Matches & Liston (1968) in investigations on irradiated fish.

The studies as a whole confirm the adequacy of a minimum dose of gamma radiation of 0·6 Mrad. for elimination of *Salmonella* from frozen meat and reveal no new microbiological hazards which would make the commercial use of the process unsafe. The recommended dose would also result in a considerable reduction in numbers of other pathogens of public health significance (Erdman, Thatcher & MacQueen, 1961*b*; Dyer *et al.* 1966). Sufficient data are now available for scrutiny by the appropriate authorities concerned with the safety of the process including those from toxicological studies on irradiated frozen meat (Hickman, Law & Ley, 1969) and investigation of methods for detecting whether meat has been irradiated or not (Hills & Smith, 1967). If approved, the process should be

considered as an alternative to the 'cooking process' described in The Meat (Sterilization) Regulations 1969.

We are grateful to Dr E. S. Anderson of the Enteric Reference Laboratory for phage typing and for the provision of a strain of *S. typhimurium* for experiments. Also, we thank Dr Joan Taylor of the Salmonella Reference Laboratory for the serological typing of salmonellas and examination of cultural characteristics.

REFERENCES

ALPER, T., GILLIES, N. E. & ELKIND, M. M. (1960). The sigmoid survival curve in radiobiology. *Nature, London* **186**, 1062.

ANDERSON, K., CROWDER, E. F. & WOODRUFF, P. (1964). The isolation of salmonellae from kangaroo meat sold as pet food. *Medical Journal of Australia* ii, 668.

BEASLEY, J., HOPKINS, G. B., MCNAB, D. J. N., RICHARDS, A. G. & KING, G. J. G. (1967). Pet meat as a potential source of human salmonellosis. *Lancet* i, 560.

BRIDGES, B. A. (1964). Microbiological aspects of radiation sterilisation. *Progress in Industrial Microbiology* **5**, 285.

COLEBY, B., INGRAM, M., SHEPHERD, H. J., THORNLEY, M. J. & WILSON, G. M. (1961). Treatment of meats with ionizing radiation. vii. Effect of low temperatures during irradiation. *Journal of the Science of Food and Agriculture* **12**, 483.

DYER, J. K., ANDERSON, A. W. & DUTIYABODHI, P. (1966). Radiation survival of food pathogens in complex media. *Applied Microbiology* **14**, 92.

ERDMAN, I. E., THATCHER, F. S. & MACQUEEN, K. F. (1961*a*). Studies on the irradiation of micro-organisms in relation to food preservation. II. Irradiation resistant mutants. *Canadian Journal of Microbiology* **7**, 207.

ERDMAN, I. E., THATCHER, F. S. & MACQUEEN, K. F. (1961*b*). Studies on the irradiation of micro-organisms in relation to food preservation. I. The comparative sensitivities of specific bacteria of public health significance. *Canadian Journal of Microbiology* **7**, 199.

GALBRAITH, N. S., TAYLOR, C. E. D., CAVANAGH, P., HAGAN, J. C. & PATON, J. L. (1962). Pet foods and garden fertilizers as sources of human salmonellosis. *Lancet* i, 372.

GRECZ, N. (1965). Biophysical aspects of Clostridia. *Journal of Applied Bacteriology* **28**, 17.

HARLAN, J. W. & KAUFFMAN, F. L. (1965). Effect of irradiation temperature and processing conditions on organoleptic properties of beef and chemical yields in model systems. Radiation Preservation of Foods. *Advances in Chemistry Series* 65, *American Chemical Society, Washington, D.C.* 1967.

HICKMAN, J. R., LAW, A. W. & LEY, F. J. (1969). Studies on the wholesomeness of irradiated meat. *Harwell Rep. no. A.E.R.E.—R-6028 (unclassified)*.

HILLS, P. R. & SMITH, A. H. (1967). An evaluation of several gas-chromatographic and colorimetric methods for the detection of irradiated horsemeat. *A.E.R.E. Rep. no. R-5461*, H.M.S.O.

HOBBS, B. C. (1965). Contamination of meat supplies. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **24**, 123.

HYNES, M. (1942). The isolation of intestinal pathogens by selective media. *Journal of Pathology and Bacteriology* **54**, 193.

IDZIAK, E. S. & INCZE, K. (1968). Radiation treatment of foods. I. Radurization of fresh eviscerated poultry. *Applied Microbiology* **16**, 1061.

LEIFSON, E. (1936). New selenite enrichment media for the isolation of typhoid and paratyphoid (Salmonella) Bacilli. *American Journal of Hygiene* **24**, 423.

LEY, F. J. (1962). The use of ionizing radiation for the treatment of raw meat. *Sanatarian, London* **71**, 185.

LEY, F. J. (1966). Application of radiation for the control of salmonellae in various foods. Food Irradiation. *Proceedings of FAO/IAEA meeting, Karlsruhe* 1966. *IAEA Pub. no.* 127, H.M.S.O.

LEY, F. J., FREEMAN, B. M. & HOBBS, B. C. (1963). The use of gamma radiation for the elimination of salmonellae from various foods. *Journal of Hygiene* **61**, 515.

LEY, F. J. & ROGERS, F. (1968). Processing with ionizing radiations. *Biochemical and Biological Engineering Science*, vol. 2, chap. 17 (Ed. by N. Blakeborough). Academic Press.

LICCIARDELLO, J. J., NICKERSON, J. T. R. & GOLDBLITH, S. A. (1968). Elimination of salmonella in poultry. Proceedings of an FAO/IAEA Panel on elimination of harmful organisms from food and feed by irradiation. *IAEA Pub. no. STI/PUB/200.* H.M.S.O.

LICCIARDELLO, J. J., NICKERSON, J. T. R., GOLDBLITH, S. A., SHANNON, C. A. & BISHOP, W. W. (1969). Development of radiation resistance in Salmonella cultures. *Applied Microbiology* **18**, 24.

MCCRADY, M. H. (1918). Tables for rapid interpretation of fermentation tube results. *Canadian Public Health Journal* **9**, 201.

MATCHES, J. R. & LISTON, J. (1968). Growth of salmonellae on irradiated and non-irradiated seafoods. *Journal of Food Science* **33**, 406.

MATSUYAMA, A., THORNLEY, M. J. & INGRAM, M. (1964*a*). The effect of freezing on the radiation sensitivity of vegetative bacteria. *Journal of Applied Bacteriology* **27**, 110.

MATSUYAMA, A., THORNLEY, M. J. & INGRAM, M. (1964*b*). The effect of freezing on the radiation sensitivity of bacterial spores. *Journal of Applied Bacteriology* **27**, 125.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of blood. *Journal of Hygiene* **38**, 732.

MOSSEL, D. A. A., VAN SCHOTHORST, M. & KAMPELMACHER, E. H. (1968). Prospects for the salmonella radicidation of some foods and feeds with particular reference to the estimation of the dose required. Proceedings of an FAO/IAEA Panel on elimination of harmful organisms from food and feed by irradiation. *IAEA Pub. STI/PUB/200.* H.M.S.O.

NORTH, W. R. (1961). Lactose pre-enrichment method for isolation of salmonellae from dried egg albumin. *Applied Microbiology* **9**, 188–95.

REPORT (1964). *Ministry of Health Report of working party on irradiation of food.* H.M.S.O.

ROLFE, V. (1946). A note on the preparation of tetrathionate broth. *Monthly Bulletin of the Ministry of Health and the Emergency Public Health Laboratory Service* **5**. 158.

STAPLETON, G. E. (1955). Variations in the sensitivity of *E. coli* to ionizing relations during the growth cycle. *Journal of Bacteriology* **70**, 357.

STATUTORY INSTRUMENTS (1967). *The Food (Control of Irradiation) Regulations* 1967. H.M.S.O.

STATUTORY INSTRUMENTS (1969). *The Meat (Sterilization) Regulations* 1969. H.M.S.O.

VAN SCHOTHORST, M. & KAMPELMACHER, E. H. (1967). Salmonella in meat imported from South American countries. *Journal of Hygiene* **65**, 321.

VERNON, E. (1969). Food poisoning and Salmonella infections in England and Wales, 1967. *Public Health, London* **83**, 205.

WITKIN, E. M. (1969). Ultraviolet-induced mutation and DNA repair. *Annual Review of Microbiology* **23**, 487.

J. Hyg., Camb. (1970), 68, 313
Printed in Great Britain

Further observations on the problem of isolating *Mycoplasma suipneumoniae* from field cases of enzootic pneumonia in pigs

By R. F. W. GOODWIN and JOAN M. W. HURRELL
Department of Veterinary Clinical Studies, University of Cambridge

(*Received* 16 *January* 1970)

SUMMARY

In previous work in this laboratory, *Mycoplasma suipneumoniae* was recovered in liquid medium from 13% of individual cases and 18% of outbreaks of enzootic pneumonia in pigs. In the work now described, however, these recovery rates, when judged by the same criteria, were 45 and 75%, respectively. As there was evidence to suggest that this second series of pneumonic cases was less suitable for cultural examination than the first series, some of the other factors that might have improved the recovery rate were investigated.

Some improvement was probably achieved by inoculating the liquid medium with three or four different dilutions of pneumonic tissue, each dilution always being in duplicate, and by incubating the inoculated tubes for over 3 weeks before discarding them.

A second advantage could have derived from the fact that all batches of liquid medium were tested for their ability to support the growth of *M. suipneumoniae* before being used to culture field material.

The effect of varying one constituent at a time was observed in controlled experiments: different batches of pig sera had a marked, variable effect on the growth of both *M. suipneumoniae* and *Mycoplasma hyorhinis*; medium made with purchased Hartley's broth was found to be superior to medium incorporating broth made in this laboratory, more so for the growth of *M. suipneumoniae* than *M. hyorhinis*; the incorporation of yeast extract made in this laboratory gave a marginal advantage for the growth of *M. hyorhinis*; and both mycoplasmas grew equally well in medium containing or lacking thallium acetate.

Some batches of medium were, by chance, markedly selective for the growth of *M. suipneumoniae* compared with *M. hyorhinis*. As the full reasons for this were not known, attempts were made to develop selective media in a more direct way. One such medium contained 5% pig serum and 15% horse serum, and a second was of similar composition, except that the pig serum used inhibited preferentially the growth of *M. hyorhinis* compared with *M. suipneumoniae*. Both media markedly favoured the growth of *M. suipneumoniae* when tested separately with cultures of *M. suipneumoniae* and *M. hyorhinis*. The second medium yielded *M. suipneumoniae* when inoculated with a 10^{-1} dilution of a culture of *M. suipneumoniae* and a 10^{-2} dilution of a culture of *M. hyorhinis*, whereas a standard batch of liquid medium, similarly inoculated with *M. suipneumoniae* did not yield this

mycoplasma until the *M. hyorhinis* culture included in the inoculum was diluted to 10^{-6}.

Both selective media, when tested on a small number of field cases, gave improved isolations of *M. suipneumoniae* compared with the routine batches of liquid medium used initially.

Considerable difficulty was experienced in producing a sufficiently high level of antibodies to *M. hyorhinis* in pig sera and to *M. suipneumoniae* in rabbit sera. This exacerbated the problem of isolating and identifying *M. suipneumoniae* from field cases of enzootic pneumonia by this cultural method.

INTRODUCTION

Goodwin, Pomeroy & Whittlestone (1965, 1967) established that enzootic pneumonia of pigs could be reproduced with a mycoplasma, which they named *Mycoplasma suipneumoniae*. Although other infective agents, especially *Mycoplasma hyorhinis* and secondary bacteria, are commonly present concurrently in the lesions from field cases, this does not invalidate the essential connexion between *M. suipneumoniae* and enzootic pneumonia: the most direct way of diagnosing the disease, therefore, is to isolate and identify *M. suipneumoniae*. Hitherto, however, this has proved difficult.

The two main media used routinely in this laboratory have been solid medium and liquid medium. With the present solid medium, *M. suipneumoniae* can rarely be recovered on primary isolation, even from experimental cases of the disease where *M. hyorhinis* and secondary bacteria are apparently absent (Goodwin, Pomeroy & Whittlestone, 1968). Using liquid medium, *M. suipneumoniae* was recovered from 91 % of such experimental cases but, in a series of 12 field outbreaks, a positive cultural diagnosis was obtained in only three of 24 cases of pneumonia (12·5 %) from two outbreaks (Goodwin *et al.* 1968). If the cultural diagnosis of enzootic pneumonia is to become a routine matter, therefore, it is essential to obtain a higher isolation rate of *M. suipneumoniae* from field material.

In later work with liquid medium in this laboratory, the percentage of successful recoveries of *M. suipneumoniae* from natural cases of the disease has been markedly increased and the purpose of this paper is to describe some of the factors that probably contributed to this more favourable situation.

MATERIALS AND METHODS

Pneumonic-lung samples

These were obtained from outbreaks of enzootic pneumonia which arose in herds that had previously shown no sign of the disease, except for the two from Herd EK, which was a chronically infected herd. The disease in all the herds satisfied the clinical and epidemiological characteristics of enzootic pneumonia (Goodwin & Whittlestone, 1967) and in nearly all the cases listed in Table 1, organisms with the morphology of *M. suipneumoniae* were seen in touch preparations examined by the method of Whittlestone (1967).

All the lung samples had been stored either at −30° C. or −60° C. before being examined culturally.

Cultural examinations

The basic liquid medium used was as previously described (Goodwin, Hodgson, Whittlestone & Woodhams, 1969). Any variations on this are described in the text. The solid medium was made from the above liquid medium as described earlier (Goodwin *et al.* 1967).

About 1 g. of pneumonic tissue was ground with sterile sand and liquid medium in a Griffith's tube; 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of lung in liquid medium were prepared, always in duplicate, for incubation. If growth occurred, further passages (usually one or two) were made in liquid medium before passaging into tubes for a metabolic-inhibition test.

In many instances, 0·02 ml. of each primary dilution in liquid medium and of later passages in this medium was seeded onto solid medium.

Serological techniques

The growth-inhibition and metabolic-inhibition tests were carried out with the same rabbit antisera (R1, R2) and in the same manner as before (Goodwin *et al.* 1968), the metabolic-inhibition tests being performed in small glass tubes (Goodwin *et al.* 1969).

RESULTS

Table 1 shows the results of culturing 45 cases of pneumonia from 16 outbreaks. The herds are listed in the order in which they were examined, as are the individual cases of pneumonia within each herd. All the serological results apply to liquid medium, for in only eight cases was a mycoplasma cultured directly on solid medium, and in none of these instances were the colonies inhibited by either the *M. hyorhinis* or *M. suipneumoniae* antiserum.

Where both *M. suipneumoniae* and *M. hyorhinis* are bracketed together, an atypical result was obtained: at first, the higher concentrations of each rabbit serum seemed to be inhibiting growth of the homologous antigen but shortly thereafter growth occurred even in these tubes. We concluded from this that both mycoplasmas were probably present in the primary inoculum, and in such a combination initially that each eventually grew through its heterologous serum.

It can be seen that *M. suipneumoniae* was recovered from 19 of the 45 cases (42 %). If the less clear-cut results are included, where the isolation of *M. suipneumoniae* appeared to be only partly inhibited by *M. hyorhinis*, the positive cases of enzootic pneumonia by cultural examination are increased to 24 (53 %). On an outbreak basis a positive diagnosis was obtained in 14 out of 16 instances. In the earlier study, however, only two cases of pneumonia were usually examined from each outbreak (Goodwin *et al.* 1968) and, in order to compare the present work more strictly, not more than the first two cases of pneumonia from each herd in Table 1 should be considered. When this is done, however, the positive diagnoses on an individual case basis become at least 13 out of 29 (45 %) and on an outbreak

Table 1. *Attempts to isolate* M. suipneumoniae *from field cases of enzootic pneumonia*

Herd	Pig	Growth in liquid medium	Serological identification
XI	983	+	*M. hyorhinis*
	984	+	*M. suipneumoniae*
	982	+	*M. suipneumoniae*
	985	+	*M. hyorhinis*
EO	989	+	NR
	991	+	*M. suipneumoniae* / *M. hyorhinis*
	990	+	*M. suipneumoniae*
	992	+	*M. suipneumoniae*
ME	998	+	*M. suipneumoniae* / *M. hyorhinis*
	999	+	*M. suipneumoniae*
UN	1003	+	*M. suipneumoniae*
	1001	+	*M. suipneumoniae*
	1002	+	NR
ZD (1)	1022	+	*M. hyorhinis*
	1023	+	*M. suipneumoniae*
	1026	+	*M. hyorhinis*
ZD (2)	1030	+	*M. hyorhinis*
	1031	+	*M. hyorhinis*
TF	1049	+	*M. suipneumoniae*
	1048	+	*M. hyorhinis*
	1050	+	*M. suipneumoniae*
LC	1051	+	DR
	1052	+	DR
	1053	+	*M. suipneumoniae*
ME	1056	+	*M. suipneumoniae*
	1057	+	*M. suipneumoniae* / *M. hyorhinis*
	1072	+	*M. suipneumoniae* / *M. hyorhinis*
	1070	+	DR
	1069	+	*M. suipneumoniae*
	1071	+	DR
DN	1076	+	*M. suipneumoniae*
TZ	1067	−	
	1068	+	*M. suipneumoniae*
DT	1058	+	*M. suipneumoniae*
	1060	−	
	1059	−	
ZC	1061	+	*M. suipneumoniae*
CI	1080	+	*M. suipneumoniae*
EK	1084	+	*M. suipneumoniae*
	1085	+	*M. hyorhinis*
TL	1063	+	DR
	1064	+	*M. hyorhinis*
	1065	+	*M. suipneumoniae* / *M. hyorhinis*
	1065A	−	
	1065B	−	

NR = no result; DR = doubtful result.

basis 12 out of 16 (75 %). As the corresponding rates of isolation in our previous study were 13 % for individual cases and 18 % for outbreaks, this is a striking improvement, and some of the possible reasons for it are considered below.

Persons making the isolations

The laboratory manipulations made in this series of attempted isolations were performed by different persons from those similarly concerned in the previous study (Goodwin *et al.* 1968). In such an event, it is always possible to obtain different results, sometimes for reasons that are not very obvious. In the present work, however, two people performed the primary inoculations and the subsequent passages, one dealing with 19 cases of pneumonia and the other with 26. Both persons isolated *M. suipneumoniae* from 42 % of their cases, and this remarkable degree of conformity suggests that factors other than personal skill were probably involved in the more frequent isolation of *M. suipneumoniae* in the work now being reported.

Selection of pneumonic samples

In the previous work (Goodwin *et al.* 1968), the pneumonic samples chosen for cultural examination were preselected, in that they were nearly all cases in which many organisms with the morphology of *M. suipneumoniae* were seen in the touch preparations.

Table 2. *Relationship between the mycoplasma recovered and mycoplasmas seen in touch preparations*

Mycoplasmas of *M. suipneumoniae* – type	Total cases	Recovery of mycoplasmas			
		M. suipneumoniae alone	*M. hyorhinis* alone	*M. suipneumoniae* + *M. hyorrhinis*	Negative or doubtful
Moderate numbers or above	20	12	2	3	3
Less than moderate numbers	25	7	9	2	7

In the present work, since the outbreaks were studied soon after they arose, there was less opportunity for selecting pneumonic cases with numerous organisms of the *M. suipneumoniae*-type in touch preparations. The effect of this is shown in Table 2. Many fewer isolations of *M. suipneumoniae*, and many more isolations of *M. hyorhinis*, were made from those cases of pneumonia where organisms of the *M. suipneumoniae*-type were sparse in the touch preparations. The isolation rate of *M. suipneumoniae* from the 20 cases that were more comparable with those examined by Goodwin *et al.* (1968) was 60 %. The more frequent recovery of *M. suipneumoniae* in the present work, therefore, was achieved despite the generally less favourable pneumonic samples.

Number of cultures put up and duration of primary incubation

In this work, several dilutions of lung were incubated in duplicate, compared with the two single dilutions (1/200 and 1/2000) used by Goodwin *et al.* (1968). Also, the primary dilutions were incubated for a longer period than previously. Because growth was often obtained in only one of a pair of duplicate dilutions and sometimes at only one particular dilution, and because growth was sometimes not apparent until 14–21 days after primary inoculation, it is likely that these later procedures contributed to the improved isolation rate.

Variability of liquid medium

Titrations of standard cultures of *M. suipneumoniae* and *M. hyorhinis* in different batches of liquid medium showed a considerable batch variability in the ability to support the growth of these organisms, particularly *M. hyorhinis*

Table 3. *Variation in growth of* M. suipneumoniae *and* M. hyorhinis *in different batches of liquid medium*

Batch of liquid medium	Highest dilution of culture producing growth	
	M. suipneumoniae	*M. hyorhinis*
1	10^{-8}	No growth
2	10^{-7}	10^{-3}
3	10^{-6}	10^{-7}
4	10^{-6}	10^{-4}
5	10^{-6}	10^{-2}
6	10^{-8}	10^{-3}
7	10^{-9}	10^{-8}
8	10^{-5}	10^{-1}
9	No growth	No growth

(Table 3). An attempt to correlate the general variability of the liquid medium with the more frequent isolations of *M. suipneumoniae* in the present work was unsuccessful; but it was known that all the batches of liquid medium used would support the growth of *M. suipneumoniae* well, whereas complete information of this type is not available for the earlier work (Goodwin *et al.* 1968). It is possible, therefore, that the chances of isolating *M. suipneumoniae* from field material can be considerably increased by using batches of liquid medium that have been pretested for the more favourable growth of this mycoplasma compared with *M. hyorhinis.*

Attempts to improve the liquid medium in favour of M. suipneumoniae

The general plan in this part of the work was to make up batches of liquid medium in which only one constituent was varied, and to test such batches together under identical conditions for their ability to support the growth of *M. suipneumoniae* and *M. hyorhinis.* In the first stage, the object was to improve the growth of *M. suipneumoniae*; thereafter, experiments were undertaken to inhibit selectively the growth of *M. hyorhinis.*

Factors influencing the growth of M. suipneumoniae

Serum. Table 4 shows the results obtained with five samples of a medium in which a different batch of pig serum had been used in each sample. It can be seen that, in general, *M. suipneumoniae* grew much better than *M. hyorhinis*, and that both these organisms grew variably in the five batches of medium. In a parallel experiment, in which four batches of pig serum, different from those listed in Table 4, were compared using *M. hyorhinis* alone, rather better growth of *M. hyorhinis* was obtained but there was still an obvious variability.

Table 4. *Variation in growth of* M. suipneumoniae *and* M. hyorhinis *in a medium made with different batches of pig serum*

	Highest dilution of culture at which growth occurred	
Batch of serum	*M. suipneumoniae*	*M. hyorhinis*
1	10^{-8}, 10^{-7}	No growth, no growth
2	10^{-5} (10^{-7}), 10^{-5} (10^{-7})	(10^{-1}), No growth
3	10^{-8}, 10^{-8}	(10^{-1}), No growth
4	10^{-7}, 10^{-7}	No growth, no growth
5	10^{-6}, 10^{-7}	(10^{-8}), No growth

Note. All titrations were made in duplicate. The figures in parentheses indicate the maximum titre of partial growth.

Hartley's broth. Four comparisons were made between two different batches of purchased Hartley's broth (Oxoid) and the broth made in this laboratory. The medium made with the purchased broth supported the growth of both *M. suipneumoniae* and *M. hyorhinis* better than the medium made with our own broth, the main difference being the much higher speed of growth in the former medium. This improvement in growth, however, was much more marked with *M. suipneumoniae* than with *M. hyorhinis.*

Yeast extract. Two comparisons were made between a purchased batch of yeast extract (DIFCO) and the yeast extract made in this laboratory. In both cases, our own yeast extract seemed slightly better for the growth of *M. hyorhinis*, but there was no growth difference with *M. suipneumoniae.*

Presence of thallium acetate. A comparison was made between medium containing the usual concentration of thallium acetate and the same medium without this inhibitor. No significant difference in the growth rate of either *M. suipneumoniae* or *M. hyorhinis* was observed with the two types of medium.

Attempts to inhibit the growth of M. hyorhinis

The first approach under this heading was to compare some variants of the standard liquid medium for their ability to support the growth of *M. suipneumoniae* more readily than the growth of *M. hyorhinis* (Table 5). Because there was sometimes a limit to the number of titrations that could be performed in parallel, these were not always taken to the limit of growth. It can be seen that during this phase of the work the standard medium containing 20 % pig serum supported the

growth of *M. hyorhinis* quite well (Expts. 1, 5), but the reduction in the percentage of pig serum in favour of horse serum depressed the growth of *M. hyorhinis*, in that this mycoplasma did not produce a complete change in pH in medium D beyond the 10^{-1}, 10^{-1} and 10^{-3} tubes, respectively, in Expts. 2, 4 and 5. Insufficient results are available for medium E to say what the effect of the complete substitution of pig serum was, but Expt. 5 indicates that the growth of *M. suipneumoniae* was partly depressed in that medium, so that the maximum difference in growth between *M. hyorhinis* and *M. suipneumoniae* which we were seeking had now been lost.

Table 5. *Comparison between five media containing varying proportions of pig and horse serum*

		Media					
Serum	Culture titrated	A	B	C	D	E	Exp. no.
Pig	—	20 %	15 %	10 %	5 %	None	
Horse	—	None	5 %	10 %	15 %	20 %	
	M. hyorhinis	10^{-7}	ND	ND	ND	10^{-6}	1
	M. hyorhinis	*10^{-3}*	*10^{-3}*	*10^{-3}*	10^{-1}	ND	2
	M. suipneumoniae	*10^{-3}*	*10^{-3}*	*10^{-3}*	*10^{-3}*	ND	2
	M. hyorhinis	*10^{-3}*	10^{-1}	10^{-1}	ND	ND	3 (passage
	M. suipneumoniae	*10^{-3}*	*10^{-3}*	*10^{-3}*	ND	ND	of Exp. 2)
	M. hyorhinis	ND	ND	ND	10^{-1}	ND	4 (passage
	M. suipneumoniae	ND	ND	ND	10^{-7}	ND	of Exp. 2)
	M. hyorhinis	*	ND	ND	10^{-3}	10^{-3}	5
	M. suipneumoniae	*	ND	ND	*10^{-8}*	10^{-5}	5

* These cultures were also titrated in parallel in a routine batch of liquid medium containing 20 % pig serum: *M. hyorhinis* gave a titre of 10^{-6}; *M. suipneumoniae* gave 10^{-7}.

ND = not done.

Note. Italicized titres mean that the titration was not taken beyond this tube. Where lines are bracketed together, all the titrations were made in parallel on the same day.

Table 6. *Mycoplasmas recovered with three selective media compared with original routine media*

		Organism recovered using each medium below		
Pig	Result from Table 1	E	D	SLM
1022	*M. hyorhinis*	*M. suipneumoniae*	*M. hyorhinis*	*M. suipneumoniae*
1048	*M. hyorhinis*	*M. hyorhinis*	*M. hyorhinis*	*M. hyorhinis*

SLM = Standard liquid medium with depressant effect on the growth of *M. hyorhinis*.

As medium D appeared to favour the growth of *M. suipneumoniae* compared with *M. hyorhinis* to a marked degree it was used, along with medium E, to re-examine two of the field cases of enzootic pneumonia (1022, 1048) in Table 1, from which only *M. hyorhinis* had been isolated previously. In addition, a batch of standard liquid medium (SLM) was included which, by chance, had the ability to depress the growth of *M. hyorhinis* (Table 6). It can be seen that media E and SLM allowed the isolation of *M. suipneumoniae* from case 1022, but *M. hyorhinis* was

still the only mycoplasma isolated from case 1048. As this was only a partial improvement, an attempt was made to prepare an even more selective medium, based on medium D, as described below.

Incorporation of serum containing antibodies to M. hyorhinis

The first difficulty in this connexion was to find pigs with sera that did not already inhibit the growth of mycoplasmas. It had previously been observed that many pig sera contained non-specific inhibitory substances (Goodwin *et al.* 1969) and we preferred to produce antisera against *M. hyorhinis* in animals that were initially free from such inhibitors, to allow the possibility of obtaining the maximum difference between the inhibition of *M. hyorhinis* and *M. suipneumoniae*. Such pigs, however, were difficult to find. A total of 23 colostrum-deprived, hysterectomy-produced pigs, and eight pigs born naturally in a herd established entirely from hysterectomy-produced pigs, were bled; the sera from all of them after inactivation (56° C. for 30 min.) had some inhibitory effect on the growth of *M. hyorhinis*, and only six of the sera (all from hysterectomy-produced pigs) had no inhibitory effect on the growth of *M. suipneumoniae*. Nevertheless, because it had previously been observed that the non-specific inhibitory effect fluctuated with time, nine of the hysterectomy-produced pigs were selected for three experiments in which they were inoculated with *M. hyorhinis*.

There were two pigs in the first experiment, four in the second and three in the third; two of the pigs in the second experiment and all three in the third experiment had preinoculation sera which did not inhibit the growth of *M. suipneumoniae*. Cultures of *M. hyorhinis* that had grown in liquid medium at high dilutions were given by various routes: intranasally (as living cultures), subcutaneously (with and without Freund's complete adjuvant), intraperitoneally and intramuscularly (with and without Freund's complete adjuvant); all the pigs received *M. hyorhinis* antigen by at least two different routes and two of the pigs were inoculated by four different routes. When the antigen was given by injection it had previously been centrifuged, washed, and resuspended in a more concentrated form in phosphate-buffered saline.

The outcome of these three separate attempts to produce a pig antiserum to *M. hyorhinis* was disappointing. All the sera were tested after inactivation and the best sample inhibited the growth of *M. hyorhinis* to a serum dilution of only 1/64, but it also partially inhibited the growth of *M. suipneumoniae* to a dilution of 1/8; furthermore, when this pig was killed 13 days later to harvest a large quantity of serum, the above titres had fallen to 1/4 (partial inhibition to 1/12), and partial inhibition to 1/4, respectively. The second point of note is that four of the five pigs that had serum with no inhibitory effect on *M. suipneumoniae* before their course of inoculations developed such an inhibitory effect afterwards (up to a serum dilution of 1/24); the single pig that did not react in this way, did not produce any inhibitory effect to *M. hyorhinis* either.

The serum sample which inhibited the growth of *M. hyorhinis* when diluted 1/64 was now incorporated into the earlier selective medium, type D. The horse-serum component in medium D avoided the problem of finding a pig serum that

did not contain inhibitory substances to *M. suipneumoniae* when diluting out such inhibitory substances in the antiserum against *M. hyorhinis*. The new medium (F) contained 6% (1/17) of the latter antiserum and 14% horse serum; it was thus only marginally different in percentage composition from the original medium D.

Titrations of *M. suipneumoniae* and *M. hyorhinis* cultures were made in medium F alone, and in both medium F and the batch of standard liquid medium (SLM) shown in Table 6, in parallel (Table 7). Medium F seemed promising, in that *M. hyorhinis* did not produce a full pH change beyond the 10^{-1} tube in all three experiments, whereas *M. suipneumoniae* grew well in the same medium; secondly, when medium F was compared with a standard liquid medium in parallel, the latter medium supported the growth of *M. hyorhinis* almost as well as *M. suipneumoniae*.

Table 7. *Titrations in selective medium (F) and in a routine batch of medium (SLM)*

Experiment	Medium	Highest dilution of culture at which growth occurred	
		M. suipneumoniae	*M. hyorhinis*
1	F	*10^{-8}*	10^{-1} (10^{-4})
2	F	*10^{-6}*	10^{-1} (10^{-3})
3	F	10^{-7} (10^{-8})	10^{-1} (10^{-2}) Titrated in
	SLM	*10^{-8}*	10^{-7} (10^{-8}) parallel

Note. Italicized titres mean that the titration was not taken beyond this tube; titres in parentheses indicate the maximum titre of partial growth.

SLM = Standard liquid medium with depressant effect on the growth of *M. hyorhinis*.

Medium F was next tested with mixed cultures of *M. hyorhinis* and *M. suipneumoniae*, but before doing this a trial experiment of this type was performed in an ordinary routine batch of liquid medium. A culture of *M. suipneumoniae* and a culture of *M. hyorhinis* were titrated in this medium, and both grew at the highest dilution tried (10^{-6}). Tubes of the same medium were put up in parallel with these titrations, each tube containing a 10^{-1} dilution of the *M. suipneumoniae* culture but with varying dilutions of the *M. hyorhinis* culture (from 10^{-1} to 10^{-6}). In all three branches of this experiment there were control tubes and every dilution was made in duplicate. The object was to see how much the *M. hyorhinis* culture had to be diluted in a mixture before it ceased to outgrow *M. suipneumoniae*. The tubes inoculated with the *M. hyorhinis* culture at the 10^{-1} to 10^{-5} dilutions yielded *M. hyorhinis* from the mixture, and only the two tubes inoculated with the 10^{-6} dilution of *M. hyorhinis* yielded *M. suipneumoniae*.

The above experiment was now repeated with medium F. The culture of *M. suipneumoniae* when titrated alone again grew at the 10^{-6} dilution (the highest dilution tried), but the culture of *M. hyorhinis* when titrated alone grew well in this medium only at the 10^{-1} dilution. There was some growth of *M. hyorhinis* at 10^{-2} and 10^{-3}, but none thereafter. With the mixed cultures (*M. suipneumoniae* at 10^{-1} throughout, and *M. hyorhinis* varying from 10^{-1} to 10^{-6}), growth occurred in both the tubes inoculated with the 10^{-1} dilution of *M. hyorhinis*, but

no result could be obtained on either of these cultures with the metabolic-inhibition test. It was concluded from this that both mycoplasmas were probably present, each growing through the heterologous rabbit antiserum. *M. suipneumoniae*, however, was recovered from all 10 tubes inoculated with *M. hyorhinis* in dilutions from 10^{-2} to 10^{-6}. It seemed, therefore, that medium F allowed the isolation of *M. suipneumoniae* from mixed cultures when it was inoculated with ten times more of a culture of *M. suipneumoniae* than of a culture of *M. hyorhinis*, whereas with the routine batch of liquid medium tried, the dose of the *M. suipneumoniae* culture had to be 100,000 times that of the *M. hyorhinis* culture before *M. suipneumoniae* was recovered.

Table 8. *Mycoplasmas recovered with two selective media compared with original routine media*

Pig	Result from Table 1	Organism recovered using each selective medium below: F	SLM
1026	*M. hyorhinis*	*M. hyorhinis*	*M. suipneumoniae*
1030	*M. hyorhinis*	*M. suipneumoniae*	*M. hyorhinis* (possibly with *M. suipneumoniae*)
1031	*M. hyorhinis*	*M. suipneumoniae*	*M. hyorhinis* and *M. suipneumoniae*

SLM = Standard liquid medium with depressant effect on the growth of *M. hyorhinis*.

Medium F was now tested with three field cases that had previously yielded only *M. hyorhinis*; at the same time, it was compared with the batch of liquid medium (SLM) shown in Table 6. The results summarized in Table 8 show that medium F allowed the isolation of *M. suipneumoniae* from both pigs 1030 and 1031; it was not greatly superior to medium SLM, however, as this gave a better result with pig 1026 and was almost successful with pig 1030.

Production of antisera in rabbits

This paper has emphasized the difficulties of isolating *M. suipneumoniae* from field cases of enzootic pneumonia. With these cultural techniques, however, there seems to be yet another difficulty, and that is the need to have good diagnostic antisera against *M. suipneumoniae* and *M. hyorhinis*. We have not been able to produce antisera to *M. suipneumoniae* very readily, despite several attempts. In all, 16 rabbits have recently been injected with concentrated antigen subcutaneously (plus Freund's complete adjuvant) followed by intravenous injections of antigen alone. In half the rabbits this course of injections was followed by intramuscular injections and intravenous injections; furthermore, in case the antigenicity of the culture of *M. suipneumoniae* had been grossly affected by the repeated centrifugation and resuspension, fresh antigen was prepared and after only one centrifugation the deposit was resuspended in one-hundredth of its original volume. The rabbits that received this unwashed antigen all developed anaphylactic shock and one of them died, but the highest serum titre produced was only 1/160.

DISCUSSION

Because *M. suipneumoniae* cannot yet be cultivated directly on solid medium from cases of enzootic pneumonia, it is necessary to make the primary isolations in liquid medium. It would seem that the isolation rate of *M. suipneumoniae* can be improved by preselecting the pneumonic tissue: active, moist cases of pneumonia with *M. suipneumoniae*-type organisms in touch preparations are likely to give better results, but the chances of success can also be improved by inoculating the liquid medium with several different dilutions of pneumonic tissue, at least two tubes being put up at each dilution. Whether the isolation rate could be improved still further by culturing from very fresh tissue which has not been frozen remains to be investigated.

The greatest problem, however, is still the blocking action of *M. hyorhinis*, when this mycoplasma is concurrently present in the lung. Some batches of liquid medium made in this laboratory appear to be much more selective than others for *M. suipneumoniae*, but this chance variation cannot be relied upon, nor can it be readily controlled. Known variations in the inhibitory capacity of normal pig serum seem to account for a large part of this over-all variability, but non-serum factors are probably also involved. Awareness of this variability could be important in those laboratories which are still having difficulty in growing *M. suipneumoniae*, even in pure culture. Most of our media will at least support the growth of *M. suipneumoniae* well, but where this organism is only being grown fitfully and in low titre, there is probably less chance of cultivating *M. suipneumoniae* in the presence of *M. hyorhinis*.

The fact that our normal liquid medium yielded *M. hyorhinis* when inoculated with 10,000 times the dose of *M. suipneumoniae* culture as *M. hyorhinis* culture indicates how formidable the task of isolating *M. suipneumoniae* from all field cases may be. The high degree of success that we have now been able to achieve, however, may mean that *M. suipneumoniae* usually vastly outnumbers *M. hyorhinis* in lesions of enzootic pneumonia, and this would explain why organisms with the morphology of *M. suipneumoniae* are usually more obvious in touch preparations.

A more selective medium than our medium F might be made if a pig serum with a much greater inhibitory effect on *M. hyorhinis* could be produced. Although we have not been very successful in this regard, other workers might find a way of producing such sera. Arising from this part of the work, it is interesting that four out of five pigs produced inhibitory substances to *M. suipneumoniae* after being injected with *M. hyorhinis*; this may indicate shared antigens or merely a non-specific stimulatory effect.

We have mentioned our difficulty in producing good antisera against *M. suipneumoniae* in rabbits because we have heard that other workers are experiencing the same problem. *M. suipneumoniae* may yet prove to be a not very antigenic mycoplasma in this context.

Although a notable improvement in isolation rate has been achieved, we have not yet reached the stage where we can regularly culture *M. suipneumoniae* from

nearly all field cases of enzootic pneumonia and, while there are many obvious ways in which this approach may be developed further, there is no way of knowing how long it might take. In the meantime, therefore, a different method of diagnosis has been studied and this will be reported separately (Goodwin & Hodgson, 1970).

We are particularly grateful to Mrs D. E. Jarvis, who provided nearly all the technical assistance. The microscopic examination of the touch preparations was made by Dr P. Whittlestone during the course of other work. Most of the expenses of this investigation, including the salary of one of the authors (J.M.W.H.), were met by a grant from the Agricultural Research Council.

REFERENCES

Goodwin, R. F. W. & Hodgson, R. G. (1970). The passive haemagglutination test for the detection of *Mycoplasma suipneumoniae* and the possible diagnosis of enzootic pneumonia of pigs. *Journal of Hygiene* **68**, 327.

Goodwin, R. F. W., Hodgson, R. G., Whittlestone, P. & Woodhams, R. L. (1969). Immunity in experimentally induced enzootic pneumonia of pigs. *Journal of Hygiene* **67**, 193.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1965). Production of enzootic pneumonia in pigs with a mycoplasma. *Veterinary Record* **77**, 1247.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1967). Characterization of *Mycoplasma suipneumoniae*: a mycoplasma causing enzootic pneumonia of pigs. *Journal of Hygiene* **65**, 85.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1968). Attempts to recover *Mycoplasma suipneumoniae* from experimental and natural cases of enzootic pneumonia in pigs. *Journal of Hygiene* **66**, 595.

Goodwin, R. F. W. & Whittlestone, P. (1967). The detection of enzootic pneumonia in pig herds. I. Eight years general experience with a pilot control scheme. *Veterinary Record* **81**, 643.

Whittlestone, P. (1967). Mycoplasma in enzootic pneumonia of pigs. *Annals of the New York Academy of Sciences* **143**, Art. 1, 271.

J. Hyg., Camb. (1970), **68**, 327
Printed in Great Britain

The passive haemagglutination test for the detection of *Mycoplasma suipneumoniae* and the possible diagnosis of enzootic pneumonia of pigs

By R. F. W. GOODWIN and RUTH G. HODGSON
Department of Veterinary Clinical Studies, University of Cambridge

(*Received* 26 *January* 1970)

SUMMARY

Fourteen cases of enzootic pneumonia, nearly all of which had presented diagnostic difficulties using the metabolic-inhibition test, were re-examined using specific pig antisera in the passive haemagglutination test (PHA). All proved positive for *Mycoplasma suipneumoniae*, indicating that the test, used in this manner, might be particularly valuable for routine diagnosis.

The PHA test was also used to demonstrate antibody to *M. suipneumoniae* in pneumonic tissue and the associated bronchial lymph nodes.

To allay our concern that cross-reactions might interfere with this and other serological tests—the complement-fixation test (CF) and precipitation in agar-gel—the specificity of our reagents and the antigenic relationships of *Mycoplasma hyorhinis*, *Mycoplasma granularum*, mycoplasma B3, *Mycoplasma hyopneumoniae* and three strains of *M. suipneumoniae* (including cloned and uncloned isolates of the J strain) were studied in various ways. Antibodies to medium constituents occurred in rabbit antiserum but did not present a problem with pig antisera. These antibodies were successfully absorbed from the rabbit antisera but it was not possible to remove medium constituents from the antigens used to produce antisera in rabbits by repeated washing.

By all these tests, the main species of mycoplasmas studied seemed to be antigenically distinct. No major antigenic differences between the three strains of *M. suipneumoniae* were revealed by the PHA test and the CF test; a slight difference in the precipitation lines of one of these strains (MG) in agar-gel might have indicated an antigenic variation or been a measure of some other factor.

INTRODUCTION

Goodwin, Pomeroy & Whittlestone (1965, 1967) induced enzootic pneumonia of pigs with a mycoplasma, which they named *Mycoplasma suipneumoniae*. In the field, other infective agents may frequently be isolated from lungs affected with enzootic pneumonia but, as respiratory diseases often become complicated in this way in many species, this does not invalidate the proposition that *M. suipneumoniae* is the primary cause of enzootic pneumonia of pigs. The most direct way of diagnosing the disease, therefore, is to isolate and identify the causal agent.

Goodwin, Pomeroy & Whittlestone (1968), using liquid medium and the metabolic-inhibition test, were able to isolate *M. suipneumoniae* from 91% of

a series of experimental cases; with the same techniques, however, they recovered *M. suipneumoniae* from only 13% of field cases, and from only 18% of field outbreaks when two specially selected cases were examined from each outbreak. Subsequently, Goodwin & Hurrell (1970) improved these figures to 45 and 75%, respectively, and developed selective media that could well allow even higher isolation rates when tested over a comparable series of field cases. Nevertheless, several technical difficulties remained: there were problems in producing good antisera in both rabbits and pigs, and there were considerable, unpredictable variations in the ability of different batches of medium to support the relative growths of *M. suipneumoniae* and *Mycoplasma hyorhinis*. It was still uncertain, therefore, whether a stage had been reached where most cases of enzootic pneumonia might be diagnosed routinely by cultural examination in liquid medium with the aid of the metabolic-inhibition test. An alternative approach to the problem using the passive haemagglutination test was therefore investigated, and the results of this study are now described.

MATERIALS AND METHODS

Preparation of antigens and antisera

Antigens were grown in liquid medium and concentrated as described earlier (Goodwin, Hodgson, Whittlestone & Woodhams, 1969*b*); they were finally resuspended in one-hundredth of their original volume, thiomersal was added (1/10,000), they were ultrasonically disintegrated for 1 min. and then stored at −20° C. Whether they were cloned or uncloned is indicated, where appropriate, in the Tables.

The history of the J strain of *M. suipneumoniae* has been summarized elsewhere (Goodwin & Whittlestone, 1963). The CZ strain of enzootic pneumonia (Goodwin *et al.* 1968) derived from a sudden field outbreak in a previously unaffected herd. The MG strain of enzootic pneumonia (R. F. W. Goodwin & P. Whittlestone, unpublished) was obtained in similar circumstances, except that the outbreak was clinically atypical, in that all the adult stock remained unaffected. This adult stock, when taken to another farm, was used to establish a herd which has never shown any signs of enzootic pneumonia over a period of several years (R. F. W. Goodwin, unpublished). A problem remains with the nomenclature of *Mycoplasma hyopneumoniae* (Goodwin *et al.* 1968): the culture given that name in this publication derived from pneumonia-inducing fluids and not from the colonies on solid medium that were so named by Maré & Switzer (1965). The 603 strain of *M. hyorhinis* (Goodwin *et al.* 1967) was used throughout. The sources of *Mycoplasma granularum* and mycoplasma B3, and the rabbit antisera R2 and R7 were as before (Goodwin *et al.* 1967). The other rabbit sera were prepared in the same general way.

The pig antisera P3018 and P3065 against *M. suipneumoniae* were from animals described by Goodwin *et al.* (1969*b*). The other two pig antisera (P3093, P3094) were obtained from animals inoculated with *M. hyorhinis* (Goodwin & Hurrell, 1970).

Serum samples were stored at about −20° C.

Serological tests

Passive haemagglutination (PHA)

This test was performed as previously described (Goodwin, Hodgson, Whittlestone & Woodhams, 1969*a*), except for the following two modifications. After trying different concentrations of tannic acid, it was found that a concentration of 1/50,000 (final concentration 1/100,000 when mixed with the suspension of sheep red cells) gave a slightly superior final suspension. Secondly, as a result of trying different times, it was found that a better antigen was obtained if the tanned red cells were sensitized for 30 min. at 37° C.

Pig antisera P3065 and P3094 were used to obtain all the results in Table 1.

Experiments on the specificity of this test have been described elsewhere (Goodwin *et al.* 1969*a*).

Complement fixation (CF)

This test was performed as previously described (Goodwin *et al.* 1969*b*), except that two units of complement were used and, with rabbit antisera, fixation was at 4° C. for 3 hr., instead of overnight. All rabbit antisera were treated overnight with an equal volume of normal pig serum (inactivated at 56° C. for 30 min.) and then centrifuged.

Precipitation in agar-gel

The double-diffusion method of Ouchterlony (1964) was used. The pig antisera used were as for the PHA test.

Isolation of mycoplasmas from pneumonic tissue and preparation of antigen

Mycoplasmas were isolated from pneumonic tissue in standard liquid medium (Goodwin *et al.* 1969*a*) as previously described (Goodwin & Hurrell, 1970). The field cases examined were from the series studied by Goodwin & Hurrell (1970). If growth was obtained, as judged by the production of acid, the culture was passaged into a larger volume (100–200 ml.) of the same medium, and the eventual antigen was harvested as previously described (Goodwin *et al.* 1969*b*). After being resuspended in phosphate-buffered saline (PBS) with 1/10,000 thiomersal, it was ultrasonically disintegrated for 30 sec. and stored at −20° C. until required.

Extraction of antibody from pneumonic tissue and lymph node

About 25–30 g. of wet pneumonic tissue was cut up finely with scissors and freeze dried. It was then ground with a pestle and mortar, although this proved rather difficult, owing to its spongy nature, and extracted with PBS, pH 8·4. After centrifugation, the supernatant fluid was dialysed against 20,000 polyethylene glycol, dialysed against saline, and adjusted to an appropriate volume.

The lymph-node extract was prepared in the same way, but from much less tissue.

RESULTS

Detection of M. suipneumoniae *and* M. hyorhinis *in pneumonic tissue*

Goodwin & Hurrell (1970) examined 45 cases of pneumonia that were believed to be enzootic pneumonia by inoculating liquid medium with dilutions of pneumonic tissue and subjecting any acid-producing agents to the metabolic-inhibition test. *M. suipneumoniae* was recovered from 19 cases; there was an atypical result, suggesting the presence of both *M. suipneumoniae* and *M. hyorhinis* in five cases; *M. hyorhinis* alone was recovered from nine cases; a doubtful result was obtained

Table 1. *Comparison of the metabolic-inhibition test, the passive haemagglutination test and precipitation in agar-gel for detecting* M. suipneumoniae *in field cases of enzootic pneumonia*

Pig	Metabolic-inhibition test*	PHA test		Precipitation in agar-gel	
		M. suipneumoniae	*M. hyorhinis*	*M. suipneumoniae*	*M. hyorhinis*
991	*M. suipneumoniae* / *M. hyorhinis*	+	+	−	+
998	*M. suipneumoniae* / *M. hyorhinis*	+	−	+	−
1022	*M. hyorhinis*	+	+	−	−
1026	*M. hyorhinis*	+	+	−	−
1030	*M. hyorhinis*	+	+	ND	+
1031	*M. hyorhinis*	+	+	−	+
1048	*M. hyorhinis*	+	+	−	+
1052	DR	+	+	−	+
1057	*M. suipneumoniae* / *M. hyorhinis*	+	+	−	+
1064	*M. hyorhinis*	+	+	−	−
1065	*M. suipneumoniae* / *M. hyorhinis*	+	+	−	−
1070	DR	+	+	−	+
1084	*M. suipneumoniae*	+	+	+	−
1085	*M. hyorhinis*	+	+	−	ND

* The results in this column are taken from Goodwin & Hurrell (1970), Table 1.

Note. The results bracketed together mean that both mycoplasmas seemed to be present in the metabolic-inhibition test, but a normal definite result was not obtained with either.

DR = doubtful result; ND = not done.

in five cases; and no result or no acid-producing agent was obtained with the remaining seven cases. One of the cases from which *M. suipneumoniae* had been identified, four of the five cases that had given a mixed result, seven of the cases that had yielded only *M. hyorhinis*, and two of the cases that had given a dubious result were examined in parallel by the PHA test and by precipitation in agar-gel. The results are shown in Table 1. The positive results in the PHA test had serum titres of 1/80 or above, whereas the corresponding titre in the single negative result was less than 1/10. In most cases, the serum was not fully titrated, the highest dilution usually being 1/320 or 1/640. However, as the

growth of the *M. hyorhinis* antigen appeared to be particularly good when the pneumonia from pig 1057 was being tested, the serum titration was taken to its limit in this case and the titre was about 1/82,000.

When the test was first performed on some of the cases listed, negative results were obtained for *M. suipneumoniae* with pigs 1022, 1030, 1048 and 1070. It was felt that this could be due to a particularly low yield of mycoplasma from the bulk culture, as growth occurred very slowly in the batch of liquid medium then being used to grow up the antigen from the primary isolation. A new batch of liquid medium was prepared, therefore, in which much better growth was obtained; the antigen prepared from this gave positive results in all four cases.

All the 14 cases in Table 1 gave a positive result for *M. suipneumoniae* in the PHA test. A positive result for *M. hyorhinis* was obtained by the same test in 13 out of the 14 cases.

As judged by precipitation in agar-gel, however, *M. suipneumoniae* was present in only 2 of the 13 cases so examined, and *M. hyorhinis* was present in only 7 of the 13 cases examined. It was concluded from this that the precipitation test was less sensitive than the PHA test, in that it probably required a greater yield of antigen for a positive result.

Detection of antibody to M. suipneumoniae *in lung tissue and bronchial lymph node*

These examinations are summarized in Table 2. The normal lung was obtained from an enzootic-pneumonia-free herd. Field case number 1 was of particular

Table 2. *Detection of antibodies to* M. suipneumoniae *and* M. hyorhinis *in tissue from the respiratory tract*

Tissue examined		Antibody titre* against *M. suipneumoniae*	Antibody titre* against *M. hyorhinis*
Normal lung		–	ND
Pneumonic tissue from field cases of enzootic pneumonia	1	640	< 4
	2	40,960	< 4
	3	> 640	< 4
	4	> 1280	< 4
	5	> 2560	< 4
Pneumonic tissue from experimental cases of enzootic pneumonia	A†	640	ND
	B‡	< 4	ND
Bronchial lymph nodes from Pig A		> 80	ND

* The figures shown are the reciprocals of the dilution.
ND = not done.
† Killed about 5 months after infection.
‡ Killed 20 days after infection.

interest: this active case of enzootic pneumonia had been repeatedly cultured by Goodwin & Hurrell (1970) in routine batches of liquid medium, and in three different types of selective medium, and had always yielded only *M. hyorhinis*

(case 1048 in Tables 1 and 6 of that publication and in Table 1 of this paper). By the PHA test, however, not only was *M. suipneumoniae* demonstrated, but also the antibody to this mycoplasma. Experimental case B was specially selected because it had been killed 20 days after infection, at a time when antibody to *M. suipneumoniae* could not be detected in its serum by the PHA test. It is of interest, therefore, that at this time antibody could not be detected in the lung either.

Possible antigenic relationships between various strains of porcine mycoplasmas

As a result of the work described above and previous studies in this laboratory (Goodwin *et al.* 1969*a*, *b*), we felt that we might be using serological methods, especially the PHA test, increasingly in the future in the diagnosis of enzootic pneumonia and for studying the epidemiology and immunology of this disease. If so, we were interested in the specificity of the test in relation to possible antigenic cross-reactions among porcine mycoplasmas. Taylor-Robinson, Somerson, Turner & Chanock (1963) had already pointed out the dangers of non-specific serological reactions when growth-medium constituents were common to the immunizing materials and the antigens used in serological tests. As it was difficult to remove contaminating growth constituents from suspensions of organisms used for immunization, these authors grew the different strains of mycoplasmas that were to be injected into rabbits or guinea-pigs in a rabbit or guinea-pig infusion medium, with corresponding rabbit or guinea-pig serum, respectively. We approached this problem another way, but likewise our main object was to satisfy ourselves that the rabbit and pig antisera used were specific.

Attempts to remove contaminating growth medium constituents by washing the antigen

A control antigen was prepared from uninoculated liquid medium and treated as described in the method for producing mycoplasma antigens. A rabbit (R 192) was injected with this control antigen, and the resulting antiserum—together with

Table 3. *Attempt to remove medium constituents by repeated washing of antigen*

Number of washings of antigen	Reciprocal of serum titre in PHA test	
	Serum R 192*	Serum P 3065†
3	10,240	5120
4	10,240	20,480
5	10,240	5120
6	10,240	10,240
7	5120	10,240
8	5120	20,480
9	2560	5120

* From a rabbit injected with medium constituents alone.

† From a pig injected with *M. suipneumoniae* antigen (J strain, uncloned) and challenged with the same strain.

Table 4. *Examination of rabbit and pig antisera using four strains of* M. suipneumoniae *and four other porcine mycoplasmas in the PHA test*

Serum	Organisms used to produce serum	Antigens used in PHA test: Control* Before absorption	Control* After absorption	*M. suipneumoniae* Strain J (uncloned)	*M. suipneumoniae* Strain CZ (uncloned)	*M. suipneumoniae* Strain MG (uncloned)	*M. hyopneumoniae* (cloned)	*M. hyorhinis* (strain 603, cloned)
R 2	*M. suipneumoniae* (strain J, uncloned)	81,920	< 20	20,480†	10,240	5120	20,480	< 20
R 7	*M. suipneumoniae* (strain J, cloned)	40,960	< 20	10,240	10,240	5120	20,480	< 20
R 360	*M. suipneumoniae* (strain J, uncloned)	ND	< 20	20,480	10,240	10,240	20,480	< 20
R 191	*M. suipneumoniae* (strain MG, uncloned)	10,240	< 20	2560	2560	5120	5120	< 20
R 211	*M. hyopneumoniae* (cloned)	20,480	< 40	5120	1280	1280	5120	20
R 11	*M. hyorhinis* (strain 603, cloned)	40,960	< 40	< 40	< 40	< 40	< 40	81,920
R 45	*M. hyorhinis* (strain 603, cloned)	20,480	< 20	< 20	< 20	< 20	< 20	20,480
R 140	*M. granularum* (cloned)	81,920	< 20	< 10	< 10	< 10	< 20	< 20
R 149	Mycoplasma B3 (cloned)	10,240	< 40	< 40	< 20	< 20	< 20	< 40
R 192	Control antigen*	40,960	< 20	2560	5120	2560	81,920	10,240
P 3018	*M. suipneumoniae*	< 20	—	20,480	10,240	10,240	10,240	< 20
P 3065	(strain J, uncloned)	< 20	—	10,240	10,240	5120	10,240	< 20
P 3093	*M. hyorhinis*	ND	—	< 20	< 20	< 20	< 20	20,480
P 3094	(strain 603, cloned)	< 20	—	< 10	< 10	< 10	< 20	20,480

* Control antigen prepared from uninoculated liquid medium.
† Serum R2 gave the same titre against the cloned, J-strain antigen. ND = not done.
Note. All figures shown are the reciprocal of the serum titre.

pig serum P3065—was tested against an antigen made from a culture of *M. suipneumoniae* in the usual way with three washings. The antigen was then washed a further six times, and after each washing and resuspension a sample was taken. All these antigenic samples were then tested against the two sera in the PHA test, and the results are shown in Table 3. It can be seen that relatively little impression on the serum titres was made by repeated washing of the antigen.

During the course of this work, two useful observations were made. First, the control antigen, when resuspended, gave a considerable opacity reading with Brown's tubes. We therefore discarded such opacity measurements; for they seemed irrelevant to the amount of mycoplasma antigen obtained from inoculated cultures. Instead, as an arbitrary standard, all final antigenic suspensions were made up to one-hundredth of the volume of the original liquid-medium culture. Secondly, we observed that if the liquid medium were centrifuged before use, *M. suipneumoniae* grew just as well in the supernatant fluid, independently of the deposit. The deposit obtained from uninoculated medium could be a consequence of storing the medium frozen but we did not pursue this possibility experimentally at this stage.

Specificity of sera

Passive haemagglutination. Three of four pig antisera were screened in the PHA test against the above control antigen for antibodies to non-porcine growth-medium components. Negative results were obtained. The four sera were then tested against *M. hyorhinis* and four strains of *M. suipneumoniae* (Table 4).

The rabbit antisera were likewise tested against the control antigen, but they had substantial titres. The titre of the control antigen had previously been established by block titration against its homologous rabbit antiserum. Each rabbit antiserum was then absorbed by adding an equal volume of either normal pig serum or freeze-dried liquid medium, or both, and holding the mixtures at 4° C. for 24 hr. After centrifugation, the clear serum was removed and tested again. Reabsorption was carried out as found necessary. These antisera were then tested against the same mycoplasmas as the pig sera, and the results are also shown in Table 4.

Complement fixation. As a further check on possible cross-reactions between *M. suipneumoniae* and *M. hyorhinis*, some comparisons were next made using the CF test (Table 5).

Precipitation in agar-gel. Finally, the antigens prepared from the J, CZ and MG strains of *M. suipneumoniae*, together with the *M. hyopneumoniae* antigen, were diffused against a standard *M. suipneumoniae* antiserum. All the antigens, except for the MG strain, shared two or more precipitation bands with each other, but only one band with the strain-MG antigen. The antigen prepared from the MG strain of *M. suipneumoniae* consistently failed to produce a strong band of precipitate which formed close to the antigen well, but shared an inner band of precipitate with the other three strains.

Table 5. *Examination of rabbit and pig antisera using* M. suipneumoniae *and* M. hyorhinis *in the CF test*

Serum	Organism used to produce serum	Antigens used in CF test: Control*	*M. suipneumoniae*	*M. hyorhinis*
R 363	*M. suipneumoniae* (J strain, uncloned)	ND	640†	320
R 363 (absorbed)		ND	640	< 20
R 360		1280	1280	640
R 360 (absorbed)		< 20	1280	< 20
R 100 (absorbed)	*M. hyorhinis* (strain 603, uncloned)	ND	< 20	5120
R 11‡ (absorbed)	*M. hyorhinis* (strain 603, cloned)	ND	80	2560
R 2‡ (absorbed)	*M. suipneumoniae* (J strain, uncloned)	ND	1280	80
P 3065		ND	640	< 20
P 3094	*M. hyorhinis* (strain 603, cloned)	ND	< 20	320

* Control antigen prepared from uninoculated liquid medium.
† All figures are the reciprocal of the serum titre.
‡ With each of these antisera, the titre of the serum control was 1/80.
ND = not done.

DISCUSSION

The difficulty hitherto in attempting to isolate *M. suipneumoniae* from pneumonic tissue has not primarily been an inability to culture acid-producing organisms in liquid medium. Goodwin *et al.* (1968) obtained such a pH change in 16 out of 23 cases examined (70%), and Goodwin & Hurrell (1970) achieved a similar result with 40 out of 45 cases (89%). The problem has arisen thereafter—in attempting to identify *M. suipneumoniae* by the metabolic-inhibition test, when more than one mycoplasma was probably present. One method of combating this difficulty is to suppress the interfering mycoplasma (usually *M. hyorhinis*), and this possibility was investigated by Goodwin & Hurrell (1970), but an alternative approach is to use a method that will recognize *M. suipneumoniae* antigen in the presence of *M. hyorhinis* antigen. The results presented here indicate that the PHA test was able to do this in a series of 14 field cases, nearly all of which had been specially selected for the difficulty they had already presented with the metabolic-inhibition test. A positive diagnosis was obtained in every case, and if these results can be confirmed on a larger number of unselected field cases it appears that cultural diagnosis could become a reliable routine procedure.

From our study of the specificity of this test and our observations on the purity of our diagnostic reagents, as judged by the CF test and precipitation in agar-gel

also, we concluded that the different mycoplasmas listed showed no major antigenic relationships, neither were any major antigenic differences noted between the different strains of *M. suipneumoniae*. A possible exception to this last statement arises from the fact that the MG strain of *M. suipneumoniae* behaved slightly differently in the precipitation test. At this stage, however, we have an open mind as to whether this represents a true antigenic difference or a variation associated with a possibly poorer yield of antigen.

Mrs D. E. Jarvis provided the main technical assistance. Most of the expenses of this work, including the salary of one of the authors (R. G. H.), were met by a grant from the Agricultural Research Council.

REFERENCES

Goodwin, R. F. W., Hodgson, R. G., Whittlestone, P. & Woodhams, R. L. (1969*a*). Immunity in experimentally induced enzootic pneumonia of pigs. *Journal of Hygiene* **67**, 193.

Goodwin, R. F. W., Hodgson, R. G., Whittlestone, P. & Woodhams, R. L. (1969*b*). Some experiments relating to artificial immunity in enzootic pneumonia of pigs. *Journal of Hygiene* **67**, 465.

Goodwin, R. F. W. & Hurrell, J. M. W. (1970). Further observations on the problem of isolating *Mycoplasma suipneumoniae* from field cases of enzootic pneumonia in pigs. *Journal of Hygiene* **68**, 313.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1965). Production of enzootic pneumonia in pigs with a mycoplasma. *Veterinary Record* **77**, 1247.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1967). Characterization of *Mycoplasma suipneumoniae*: a mycoplasma causing enzootic pneumonia of pigs. *Journal of Hygiene* **65**, 85.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1968). Attempts to recover *Mycoplasma suipneumoniae* from experimental and natural cases of enzootic pneumonia in pigs. *Journal of Hygiene* **66**, 595.

Goodwin, R. F. W. & Whittlestone, P. (1963). Production of enzootic pneumonia in pigs with an agent grown in tissue culture from the natural disease. *British Journal of Experimental Pathology* **44**, 291.

Maré, C. J. & Switzer, W. P. (1965). New species: *Mycoplasma hyopneumoniae*—a causative agent of virus pig pneumonia. *Veterinary Medicine* **60**, 841.

Ouchterlony, Ö. (1964). Gel-diffusion techniques. In *Immunological Methods*. Oxford: Blackwell Scientific publications.

Taylor-Robinson, D., Somerson, N. L., Turner, H. C. & Chanock, R. M. (1963). Serological relationships among human mycoplasmas as shown by complement-fixation and gel diffusion. *Journal of Bacteriology* **85**, 1261.

J. Hyg., Camb. (1970), **68**, 337
Printed in Great Britain

Observations on the nasopharyngeal carriage of *Haemophilus influenzae* type b in children in Kampala, Uganda

By Y. MPAIRWE

Department of Medical Microbiology, Makerere University College, Kampala, Uganda

(*Received* 26 *January* 1970)

SUMMARY

Haemophilus influenzae type b was isolated from 4·5% of outpatient children living in various parts of Kampala city and its surroundings. In contrast, this serotype was carried by up to 53% (average 29%) of 14 to 18 children living as a group in an orphanage. This finding indicates that the high carriage rate for this serotype demonstrated by Turk (1963) in a group of orphanage infants in Jamaica was not an isolated finding, and that it may be expected where large groups of children live together.

H. influenzae type b did not appear to be a readily transmitted organism even in that group of children with a high carriage rate. This suggests that in ordinary open communities the transmission of this serotype from one household to another may be an extremely rare event.

INTRODUCTION

Turk (1963), working in Jamaica, showed that up to 70% of infants living together in an orphanage could carry *Haemophilus influenzae* type b in their nasopharynx without any apparent illhealth that could be attributed to this serotype. This carriage rate contrasted sharply with that of less than 5% in children in ordinary open communities (Dawson & Zinnemann, 1952; Masters, Brumfitt, Mendez & Likar, 1958; Turk, 1963). It is not known whether the high carriage rate found in the Jamaican infants was peculiar to that study or whether it is to be expected in similar groups of children in other parts of the world. The purpose of this paper is to report that an essentially similar situation existed in a group of orphanage children in Kampala. Observations on the communicability of *H. influenzae* strains are also reported.

MATERIALS AND METHODS

Two populations of children were investigated. The first consisted of children in Sanyu Babies' Home, an orphanage in Kampala, and the second of children attending an outpatient clinic in Mulago Hill Dispensary, Kampala.

The children in the orphanage lived in three separate groups that will be referred to as I, II and III. Group I children were infants confined to their cots for most of

the time. Most of the new admissions to the Home went into this group. Group II consisted of older children, most of whom were toddlers, often promoted from group I. Children in this group mixed freely with one another. The rooms occupied by these two groups were adjacent to each other on the same building but with no direct communication between them so that children in these two groups rarely mixed. Children in group II were however always brought into group I room for swabbing, at all swabbing sessions. They spent most of their day time playing in the garden a few yards outside group I room. Group III children lived in a separate house, about 50 yards away and were usually promoted from group II. The three groups were looked after by the same nurses who moved freely between these groups.

Per-nasal swabs were collected at intervals of between 1 and 4 weeks between mid-July 1967 and the last week of April 1968, except that group III children were not swabbed after the end of 1967.

Similar swabs from the outpatient children were collected on various dates between 20 May 1968 and 27 June 1968 inclusive and on each occasion between 10 and 60 consecutive subjects aged between 1 week and 10 years were swabbed. Altogether 198 children from 184 families living in the city or the neighbouring villages were investigated.

The method of collecting the nasopharyngeal swabs, of culturing them and of identifying and typing the *H. influenzae* strains were similar to those used by Turk (1963) except that the horse-blood-agar plate was omitted in this study.

FINDINGS

Carriage rates and serotypes

The orphan home. The carriage rates of *H. influenzae* strains in the orphanage groups are shown in Table 1. A hundred and twenty-one typable strains were isolated from the total of 646 swabs collected from this home over the $8\frac{1}{2}$ month period; they all belonged to type b. No case of *H. influenzae* type b infection occurred in this home during the period of the survey.

Outpatient children. The numbers of strains of different serotypes and of untypable strains isolated from the outpatient children are shown in Table 2. *H. influenzae* type b was isolated from nine (4·5%) of the 198 children.

The interval between admission and acquisition of H. influenzae *strains by children in the orphanage*

Untypable strains. There were 26 children who were admitted to group I after the study had commenced. Seven of these carried untypable strains on their first swabbing and were presumably carriers of these strains when admitted. Fifteen others, who apparently were not carriers of any *H. influenzae* strains on admission, became carriers of untypable strains within 20 weeks of admission (13 of them within 12 weeks). Only four children never became demonstrable carriers of any *H. influenzae* strains. These four had been in the home for only 4 to 10 weeks when the study was concluded.

H. influenzae *type b.* In contrast, only one child out of the 26 picked up a type b

strain while residing in group I, and of the seven who were subsequently promoted to group II where the carriage rate of this serotype varied between 6 and 53 % (average 29 %) only two became demonstrable carriers of this serotype within 6 weeks of their residence in group II. The other five apparently did not acquire this serotype although four of them had stayed in this group for 8 to 24 weeks before the study was concluded. All of the seven promoted children had acquired untypable strains while in group I.

Table 1. *Nasopharyngeal carriage of* Haemophilus influenzae *in children in Sanyu Babies' Home, Kampala*

Group	No. of children per batch	No. of batches	No. of swabs taken	Average age in months	Percentage mean carrier rate for type b	Percentage mean carrier rate for All strains
I	6–12	22	207	$5\frac{3}{4}$ ($\frac{1}{4}$–12)	7 (0–33)	54 (9–100)
II	14–18	22	346	15 (4–84)	29 (6–53)	80 (40–100)
III	4–7	16	93	24 (4–48)	4 (0–25)	53 (17–83)

The figures in parentheses indicate the ranges of age, or of percentage carrier rate.

Table 2. *The carriage rates of* Haemophilus influenzae *strains in outpatient children*

Age group	No. of subjects examined	Typable strains a	b	c	d	e	f	Untypable strains
1 week–3 months	27	—	—	—	—	1	—	13 (48)
> 3–12 months	56	—	4	—	1	2	—	31 (55)
> 1–2 years	57	—	4	—	—	2	2	34 (60)
> 2–5 years	41	—	1	—	1	—	—	27 (66)
> 5–10 years	17	—	—	—	—	—	—	12 (71)
Totals	198	—	9	—	2	5	2	117 (59)

Figures in parentheses refer to percentage for that age group.

DISCUSSION

H. influenzae is carried in the upper respiratory tract of a large proportion of healthy subjects (Turk & May, 1967). In this site in open communities, *H. influenzae* type b is carried by about 3 % of children under 5 years of age and by about 1 % or less of older subjects (Dawson & Zinnemann, 1952; Masters *et al.* 1958; Turk, 1963). The 4·5 % carriage rate of this serotype in the outpatient children investigated in the present study is consistent with these findings.

On the other hand in the orphanage infants surveyed by Turk (1963) a carriage rate of between 17 and 70 % (average 47 %) for this serotype was found. In the present study the carriage rate for this serotype in group II children varied between 6 and 53 % and averaged nearly 30 %, and possibly a higher carriage rate

might have been demonstrated if, as in Turk's study, a blood agar plate had been used in addition to the 'chocolate' agar for primary isolation. Johnson & Fousek (1943) demonstrated a 54 % carriage rate in 13 children up to 10 years of age living in one ward of a convalescent hospital. In an adjacent ward consisting of 17 'older children' no nasopharyngeal carriers of this serotype were found. All of this suggests that where small children live together in fairly large numbers the tendency is for the carriage rate of this serotype to rise to a much higher level than that found in ordinary open communities. That this is not always the case is shown by the low carriage rates in groups I and III children in my study. In group III this might have been due to acquired immunity arising out of prior contact with the serotype as most of the children in this group were former residents of group II.

The difference in carriage rates of this serotype in the groups I and II is worthy of comment; it existed in spite of the following facts.

(*a*) The two groups of children lived adjacent to each other in the same building and group II children always played in the garden a few yards outside group I room from where infected droplets could have been conveyed to group I children through open windows by means of air currents.

(*b*) The two groups were looked after by the same nurses who moved freely between the two groups and could have communicated this organism from group II to I.

(*c*) Group II children were always swabbed in group I room at all of my swabbing sessions at intervals of between 1 and 4 weeks, on which occasions the carriers in group II presumably showered large numbers of infected droplets into the air as they screamed.

The existence of the difference in the carriage rates, therefore, suggests that as means of communicating *H. influenzae* type b air currents, adult contacts and occasional entry of carriers into the environment of a susceptible community are unimportant factors. The fact that group I children were generally younger than those in group II (Table 1) might be thought to be the explanation for the difference in the carriage rates but this seems to be unlikely because even premature newborn infants have been known to carry *H. influenzae* type b in very high numbers (Donald & Coker, 1957).

Turk (1963) observed, in the orphan home he investigated, that there was always a time lag, often of 2 to 3 months, and sometimes longer, between the arrival into the home and the demonstration of type b in the nasopharynx of the new entrants. This observation, suggesting that even where the carriage rate of this serotype was high the transmission of this serotype to susceptibles did not occur readily was made in the present study also. Of the seven children that were promoted from group I to II during the period of my study apparently only two became carriers of this serotype within 6 weeks of residence in the new group. The others did not seem to have acquired this serotype although nearly all of them had been in this group for 2 to almost 6 months when the study was concluded. These observations suggest that in ordinary open communities, where contact between carriers and susceptibles is not so common or so prolonged as it was in these orphanages and where the carriage rate of this serotype is very much lower,

the transmision of *H. influenzae* type b from one household to another must be a very rare event.

I am grateful to Professor E. Nnochiri for having read the draft manuscript and to Mrs F. Mulira for permission to swab the children in Sanyu Babies Home.

REFERENCES

Dawson, B. & Zinnemann, K. (1952). Incidence and type distribution of capsulated *H. influenzae* strains. *British Medical Journal* i, 740.

Donald, W. D. & Coker, J. W. (1957). The role of *Hemophilus influenzae* in respiratory infections of premature infants. *American Journal of Diseases of Children* **94**, 272.

Johnson, R. D. & Fousek, M. D. (1943). A study of the spread of *H. influenzae*, type b. *Journal of Bacteriology* **45**, 197.

Masters, P. L., Brumfitt, W., Mendez, R. L. & Likar, M. (1958). Bacterial flora of the upper respiratory tract in Paddington families, 1952–4. *British Medical Journal* i, 1200.

Turk, D. C. (1963). Naso-pharyngeal carriage of *Haemophilus influenzae* type b. *Journal of Hygiene* **61**, 247.

Turk, D. C. & May, J. R. (1967). Distribution of *Haemophilus influenzae* in healthy human communities, in *Haemophilus influenzae, its Clinical Importance*. London: English Universities Press, p. 13–23.

The Journal of Hygiene Volume 68, No. 2 June 1970

CONTENTS

Printed in Great Britain at the University Printing House, Cambridge