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Studies on the carrier state of cattle exposed to foot-and-mouth disease virus

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The experimental and epizootiological evidence for the existence of a carrier state in cattle following exposure to foot-and-mouth disease virus (FMDV) has been reviewed by van Bekkum, Frenkel, Frederiks & Frenkel (1959). These workers also reported their own work which showed that virus could be detected regularly in the 'saliva' from a rather large proportion of cattle over a period of several months after recovery from clinical disease. These findings have been confirmed by Sutmöller & Gaggero (1965) during the course of studies undertaken to develop suitable techniques for recovery of virus from field outbreaks of disease when vesicular material was no longer available. Both groups of workers used a sampling instrument to collect a fluid specimen from the upper part of the oesophagus, pharynx and mouth. Van Bekkum *et al.* have referred to such specimens as 'saliva'; Sutmöller & Gaggero have preferred the term 'oesophageal fluid'.

The studies reported here were concerned with the measurement of the infectivity of samples taken by this method and the determination of the sites of virus persistence and multiplication in the carrier animal.

MATERIALS AND METHODS

Virus strains

(1) A-119: Pirbright stock cattle strain used at the 25th and 26th cattle passage.
(2) A-Turkey 19/64
(3) SAT 1, SA. 13/61
(4) SAT 3, Bec. 3/64
} World Reference Laboratory samples received from the field and used at the 1st to the 3rd cattle passage.

Cattle

The supply and maintenance of cattle under experiment have been described by Henderson (1952). Cattle retained for periods longer than 6 weeks after exposure to virulent virus were washed thoroughly and moved to a clean Isolation Unit.

The previous experimental history of these cattle was such that they could be placed in one of two categories:

A: Animals which had been vaccinated with inactivated FMD virus vaccines and then challenged 20–21 days later by tongue inoculation of $10^{5.0}$ ID_{50} of virulent virus. The majority of these animals had possessed sufficient immunity to modify the development of local lesions considerably and to prevent the development of secondary lesions completely.

B: Animals which had developed generalized FMD. These had been used for the passage or titration of the above virus strains or challenged as susceptible controls in vaccine evaluation trials.

Collection and handling of samples

Food was withheld from the animals for 18 hr. before taking samples.

Saliva

Saliva was collected by means of a disposable syringe from the region between the lateral surface of the tongue and the mandibular cheek teeth.

Oesophageal/pharyngeal sample

The collecting beaker or cup referred to by van Bekkum *et al.* (1959) and recently described by Sutmöller & Gaggero (1965) was passed by mouth into the oesophagus for a distance of about 6 in. As it was being withdrawn, attempts were made to contact both lateral walls of the pharynx. The volume of the sample collected varied from 5 to 10 ml. and consisted mainly of saliva. Varying amounts of flocculating mucus and cell debris were present and on occasion small hair-balls and traces of regurgitated rumen contents. Immediately after collection each sample was added to an equal volume of diluent and the mixture shaken vigorously.

Post-mortem specimens

Superficial scrapings were made of the surface epithelium from 2–12 sites from each animal. The areas sampled are described in the terminology used by Sisson & Grossman (1945) and included the dorsal and ventral turbinates and the posterior part of the nasal septum; the anterior part of the dorsum, the dorsal prominence and the lateral surface of the tongue; the glosso-epiglottic space and the root of the tongue; the lateral and dorsal surfaces of the pharynx; the dorsal and ventral surfaces of the soft plate; the glandular, cervical and thoracic regions of the oesophagus; the cervical and thoracic regions of the trachea; and the wall of the urinary bladder. In addition, the contents of the tonsillar sinuses and the bulk of the lateral and central zones of each tonsil were collected from the majority of animals.

No attempt was made to clean or wash the tissues of the first six animals examined *post mortem* but thereafter the tissues were washed thoroughly in running water before sampling began and after taking each specimen.

Each specimen was added to a standard volume of diluent which was later increased if necessary to give a final dilution of 1/10 (v/v) of the original harvest as estimated by a packed cell volume. The scrapings of epithelium and the contents of the tonsillar sinuses were processed by grinding in a Griffith's tube and the resultant suspension assayed for infectivity. The tonsils were ground with sand and only the supernatant fluid screened for infectivity.

All samples were held at room temperature and assayed for infectivity within 2–3 hr. of collection or of the slaughter of the animal.

Diluent

Phosphate buffered saline (PBS) was used as a diluent for all purposes. Antibiotics were added to give final concentrations of penicillin 1000 units/ml., streptomycin 400 μg./ml., polymyxin B 100 units/ml., neomycin 140 μg./ml. and mycostatin 100 units/ml.

Isolation and infectivity assay of virus

Plaque assay

Samples were examined for plaque-forming units in baby hamster kidney (BHK) cell strain monolayers (Mowat & Chapman, 1962); 0·2 ml. volumes of the sample were placed on 5–8 BHK 2-day-old monolayers in 60 mm. petri dishes and after an adsorption period of 1 hr. at 37° C. a nutrient agar overlay was added. The monolayers were incubated for 48 hr., stained with a 1/10,000 dilution of neutral red in PBS and plaques were counted. When recovery of the virus was required for confirmation of type or other tests a 1/20,000 dilution of neutral red in a nutrient agar overlay was used, plaques were picked and the virus grown up in BHK bottle cultures.

Mouse inoculation

Groups of 20–30 6-day-old randomized Pirbright-strain mice were inoculated intraperitoneally with 0·1 ml. volumes of the sample. Mice were observed for 8 days and mice dying after 24 hr. were checked for complement-fixing virus antigen either directly or after a further passage.

Serum neutralization tests

The cell metabolic inhibition test (colour test) using primary monolayers of pig kidney cells (Martin & Chapman, 1961) was used. The neutralization titres are expressed as the log. reciprocal of the final dilution of serum present in the serum virus mixture at the 50% end-point (estimated according to the method of Kärber, 1931). Homologous tissue culture adapted virus strains were used in these tests.

RESULTS

The infectivity of oesophageal/pharyngeal samples

Details of the frequency of recovery of virus and the titres measured in samples collected from a group of ten cattle after infection with the A-119 virus are shown in Table 1.

The figures presented are those obtained from plaque counts in BHK monolayers. The presence of virus in the majority of these samples was confirmed by recovery of virus in mice. Occasionally, samples with a high infectivity in BHK cells were negative in mice and, conversely, samples negative in BHK cells proved positive in mice. In general, samples with a plaque count of up to 5/ml. killed up to 15% of mice inoculated, samples with counts of 15–50/ml. killed on average 40%, and samples with plaque counts greater than 100/ml. killed 80–100% of mice. Any comparison of the sensitivity of the two methods used for isolating virus

must be related to the volumes of the sample tested. As a routine, 2·0 ml. of each sample were inoculated into mice and 1·0 ml. into BHK monolayers. Another factor which would affect sensitivity is the ability of the assay system to detect partially neutralized virus. Hyslop (1965) has shown that neutralization indices (measured in mice) of saliva collected from challenged animals can range from 1·19 to 3·7. No direct experiment was made to estimate the neutralizing activity

Table 1. *The frequency of recovery of virus and the infectivity of oesophageal/pharyngeal samples from ten cattle convalescent after clinical infection with FMD virus A-119*

Weeks after infection	No. of cattle yielding positive samples	Infectivity of samples	
		Mean	Range
2	9	2·1*	1·3–3·0
3	10	2·4	1·7–3·0
4	10	2·1	1·3–2·9
5	10	2·0	1·2–2·9
6	9	1·8	1·2–2·9
9	9	1·3	0·6–2·0
10	7	1·4	0·3–1·8
11	9	1·3	0·3–2·4
12	8	0·9	0·3–2·0
13	7	1·5	0·8–1·9
14	7	1·2	0·3–2·1
17	7	1·4	0·9–1·8
19	6	1·4	0·9–1·8
22	5	0·8	0·3–1·9
26	5	1·0	0·3–2·0

* Log_{10} pfu/ml. of undiluted sample.

Table 2. *Recovery of virus from oesophageal/pharyngeal samples collected from cattle convalescent after clinical infection with FMD virus strains*

Virus	Period of sampling: weeks after infection	No. of occasions sampled*	No. of cattle in group	No. of samples from which virus recovered	Comments
A-Turkey	3 to 21	12	4	40	1 animal yielded virus on all occasions, 3 animals intermittently
SAT 1	2 and 3	2	4	8	All animals yielded virus on both occasions
SAT 3	14 to 25	9	6	9	Only 3 animals yielded virus during this period
SAT 3†	2 to 8	7	6	33	3 animals yielded virus on all occasions, 3 animals intermittently

* Intervals between sampling varied from 1 to 3 weeks.
† Virus recovered from carrier cattle 14 and 18 weeks after infection.

of oesophageal/pharyngeal samples in mice or BHK monlayers. However, the results of repeat titrations in BHK cells of samples after storage at 4° C. showed a five-fold reduction in titre to have occurred after 2 days storage (mean of ten samples) and a 20-fold reduction in titre after 7 days (mean of 7 samples). This loss in infectivity may have resulted from the presence of low levels of neutralizing antibody in the samples or from inactivation due to the high pH of these samples (mean pH $8{\cdot}24 \pm 0{\cdot}16$).

Similar statistics to those shown in Table 1 were collected for four other groups of cattle after infection with three different strains of virus. This work is summarized in Table 2. The results given in Tables 1 and 2 indicate that these strains of FMDV can persist and multiply in the majority of cattle for an extended period following clinical infection. The infectivity of samples collected from the SAT 3 and A-Turkey cattle remained fairly low and only on two occasions were samples collected which contained 100 pfu/ml. In contrast, some of the animals convalescent to the A-119 strain yielded samples between the 2nd and 6th week with infectivities up to 1000 pfu/ml.

Van Bekkum *et al.* (1959) reported that vaccinated cattle exposed to virulent virus could also become carriers. This was confirmed in a group of cattle which had been challenged 20 days after vaccination with an inactivated A-119 vaccine. Twenty of 21 animals sampled 2 weeks after challenge yielded positive samples (mean infectivity—$\log_{10}$ pfu/ml. 2·1, range 0·9–3·0) 8 of 8 at 3 weeks (2·2, range 1·3–2·8) and 5 of 5 at 4 weeks (2·0, range 1·6–2·6). The geometric mean pre-challenge antibody level of these cattle was 1·69 and their immune status sufficient to prevent the development of secondary lesions following challenge. These figures found for the immune animals are similar to those for fully susceptible cattle (Table 1). Thus, the possession of immunity at the time of challenge would not appear to reduce the incidence or lessen the intensity of the carrier state.

Sites of multiplication of virus in the carrier animal

The recovery of virus in relatively high titre from carrier animals over several months must indicate continual multiplication of virus at some site or sites in the animal. The high serum antibody levels of these animals suggest that the source of infectivity is in direct or indirect communication with the upper digestive and respiratory tracts. Some workers, e.g. Cottral, Gailiunas & Campion (1963), have interpreted the results of van Bekkum *et al.* (1959) as suggesting that the source of the virus was the salivary glands. Saliva samples taken from the mouth of eighteen animals convalescent after infections with three of the virus strains used in these studies were all negative for virus when assayed in BHK cells, but oesophageal/pharyngeal samples taken immediately afterwards were all positive. Hyslop (1965) also failed to detect infectivity in saliva samples taken from the mouths of twelve steers 5 weeks after infection. On the basis of these findings, no examination of the salivary glands for virus was undertaken in the present series of experiments. Cottral *et al.* (1963) studied the persistence of virus in lymph nodes of the heads and carcases of cattle following infection with FMDV. They obtained some evidence that virus persisted for up to 15 days but not for 17 days after infection.

P. Sutmöller (personal communication), failed to detect virus in the salivary glands and the lymph nodes of a carrier bullock slaughtered 1 month after challenge. These reports did not indicate that the lymph nodes associated with the upper digestive and respiratory tracts were responsible for the virus detected in oesophageal/pharyngeal samples from carrier animals and lymph nodes were not therefore examined.

The results of infectivity titration of post-mortem specimens from fourteen cattle killed 3–5 weeks after challenge with the A-119 virus are detailed in Table 3. These animals, with the exception of FE 48 and FE 49, had been vaccinated with an inactivated A-119 virus preparation 20 days before challenge. The infectivity titres of oesophageal/pharyngeal samples taken from three of these animals immediately before slaughter have been included in Table 3, as also have the neutralizing antibody titres of the pre-challenge and post mortem serum samples. Virus was recovered from thirteen of the fourteen animals.

The results obtained from forty more cattle killed between 2 and 18 weeks after challenge or infection are summarized in Table 4. Virus was recovered from 18 of 22 cattle killed 2 to 3 weeks after challenge with SAT 1 virus, 4 of 6 animals killed 3 weeks after challenge with A-Turkey virus, 5 of 6 cattle killed approximately 10 weeks and 1 of 6 killed 28 weeks after infection with SAT 3 virus.

The percentage frequency of virus recovery and the mean infectivity levels measured for the positive specimens collected from the forty-one cattle from which virus was recovered *post mortem* are given in Table 5.

These figures were compiled from infectivity assays in BHK cells. The results of mouse inoculation did not support these figures. Samples from the pharynx and dorsal surface of the soft palate which gave infectivity levels of up to 1000 pfu/ml. killed only a small percentage of mice inoculated. This can be explained by the ability of the mouse to detect minimal levels of antibody. It was found that supernatant fluids of suspensions prepared from pharyngeal scrapings from carrier animals possessed considerable neutralizing activity. When the homologous virus was added to such fluids and incubated at 37° C. for 1 hr., infectivity was reduced 30-fold when measured in BHK and up to 5000-fold when measured in mice. Similar tests with these tissue supernatants using a virus of different immunological type resulted in no reduction in infectivity for BHK and only a fourfold drop in infectivity when titrated in mice.

The results indicate that the mucosae of the pharynx and the dorsal surface of the soft palate (which forms the anterior floor of the pharynx) are the main sites of virus multiplication in the carrier animal. Other regions contiguous or in close proximity to these areas also yielded virus on occasion but at much lower titres. Whether these recoveries represent additional sites of virus multiplication is debatable. Attempts to relate the amount of virus recovered from any particular site with the importance of that site to the carrier state must take account of the nature of the specimen assayed. Specimens from the pharynx and dorsal surface of the soft palate contained the bulk of the surface epithelium and also some sub-epithelial lymphoid tissue. Such specimens possessed considerable neutralizing activity and the infectivity levels recorded for these sites were almost certainly

Table 3. *Infectivity titration of specimens obtained from cattle* 21–37 *days after challenge with A*-119 *virus*

Days after challenge...	21		23		28		29	31		35			37	
Cattle no. (prefix FE)	23*	26*	2*	20*	6*	19*	24	28	98	30	48	49	90	99
Oesophageal/pharyngeal sample (*ante mortem*)†	NT	NT	NT	NT	1·8	1·8	2·3	NT	NT	NT	NT	NT	NT	NT
Tongue	0·0	1·0	0·0	1·4	NT	NT	NT	NT	NT	0·0	0·0	0·0	0·0	0·0
Glosso-epiglottic space	0·0	1·7	0·0	0·0	0·0	1·6	0·8	0·0	1·1	0·0	0·0	0·0	NT	NT
Pharynx	0·0	1·9	2·3	1·1	2·6	3·0	2·7	2·0	2·9	0·0	2·2	2·1	0·0	2·0
Dorsal surface soft palate	NT	NT	1·8	2·1	2·7	3·2	2·5	2·8	2·7	1·0	2·2	1·7	2·7	2·2
Ventral surface soft palate	NT	NT	1·9	0·0	1·6	1·6	0·8	0·0	0·0	0·0	0·0	0·0	NT	NT
Tonsillar sinuses	0·0	1·3	1·1	0·0	1·4	0·0	1·2	1·9	0·0	0·0	NT	0·0	NT	NT
Tonsils	NT	1·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	NT	NT
Glandular region—oesophagus	0·0	0·0	0·0	0·0	2·5	2·1	0·0	0·0	1·1	0·0	0·0	0·0	0·0	0·0
Remainder of oesophagus	0·0	0·0	0·0	0·0	1·1	0·0	0·8	0·0	0·0	NT	NT	NT	NT	NT
Turbinates	NT	NT	NT	NT	0·0	0·0	0·0	NT	NT	NT	NT	NT	NT	NT
Trachea	0·0	0·0	2·0	0·0	0·0	0·0	0·0	0·0	0·0	NT	NT	NT	NT	NT
Urinary bladder	NT	NT	NT	NT	NT	NT	NT	NT	NT	0·0	0·0	0·0	NT	NT
Pre-challenge serum titre‡	1·8	1·5	1·7	1·9	1·5	2·0	1·5	1·8	2·0	2·3	0·6	0·5	1·7	1·5
Post-mortem serum titre‡	3·0	3·7	3·7	4·2	NT	NT	3·7	3·0	3·1	3·6	3·0	3·0	3·1	3·3

* Tissues not washed before collection of specimens.
† Log_{10} pfu/g. or ml. of undiluted specimens.
‡ Log reciprocal serum dilution.
NT, Not tested.

considerably underestimated. Specimens taken from the tongue, glosso-epiglottic space and oesophagus contained less cellular material and possessed relatively little neutralizing activity. The irregular occurrence and low levels of virus recovered from these areas could represent contamination from the pharyngeal region.

Table 4. *Frequency of virus recovery and mean infectivity of specimens taken* post mortem

Virus...	SAT 1		A-Turkey		SAT 3			
Number of cattle	22		6		6		6	
Days after infection	14–21		21		62–75		196	
Tongue	3/22*	1·7†	NT	—	0/2	—	NT	—
Glosso-epiglottic space	8/22	1·9	1/3	1·0	0/4	—	NT	—
Pharynx	11/22	1·5	4/6	2·2	4/6	1·8	0/6	—
Dorsal surface soft palate	18/22	2·4	4/6	2·3	4/6	2·0	1/6	1·6
Ventral surface soft palate	8/22	1·6	NT	—	1/2	1·0	NT	—
Tonsillar sinuses (2)	4/44	1·6	2/12	1·1	0/12	—	0/12	—
Tonsils (2)	NT	—	0/12	—	0/12	—	0/12	—
Glandular region oesophagus	2/22	1·4	1/6	1·0	0/2	—	NT	—
Remainder of oesophagus	NT	—	NT	—	0/2	—	NT	—
Turbinates	NT	—	NT	—	0/2	—	NT	—
Trachea	4/22	1·3	1/3	1·0	0/2	—	NT	—
Urinary bladder	NT	—	NT	—	0/2	—	NT	—

* No. of positive specimens/no. of specimens tested.
† Mean infectivity of positive specimens ($\log_{10}$ pfu/g. or ml. of undiluted specimen).
NT, Not tested.

Table 5. *Percentage frequency of virus recovery and mean infectivity of specimens collected from forty-one cattle from which virus was recovered* post mortem

Specimen	Number examined	Percentage frequency virus recovery	Mean infectivity of positives
Dorsal surface of soft palate	40	97	2·26*
Pharynx	41	73	1·87
Ventral surface of soft palate	30	43	1·49
Glosso-epiglottic space	36	36	1·66
Tonsillar sinuses	38	29	1·43
Tongue	25	20	1·52
Trachea	30	20	1·37
Glandular region of oesophagus	35	17	1·60
Remainder of oesophagus	10	20	0·95
Tonsils	21	5	1·00

* $\log_{10}$ pfu/g. or ml. of undiluted specimen.

DISCUSSION

The results presented, in conjunction with the findings of van Bekkum *et al.* (1959) and Sutmöller & Gaggero (1965), suggest that the carrier state in cattle may be a normal sequel to infection with virulent strains of FMDV. The four virus strains

used in these investigations were examples of differing epizootiological origin. The SAT 3 strain was recovered from a very mild outbreak of disease in Bechuanaland and only produced minor clinical lesions in a small proportion of animals at risk (R. S. Hedger, personal communication). The virus, however, proved to be fully virulent for cattle in tests at this Institute. The SAT 1 strain was recovered from the 1961 enzootic in South Africa, some details of which have been recorded by Galloway (1962). The A-Turkey virus was recovered from the 1963–65 epizootic in the Middle East which resulted in a severe, widespread infection of cattle. The A-119 strain can be classed as a virulent laboratory strain in that it has been maintained by passage in cattle since its recovery from a British field outbreak in 1932.

The superficial nature of the virus infection of the pharyngeal region of carrier animals, together with the relatively high infectivity found in some samples taken from the living animal, must constitute a potential hazard to susceptible animals maintained in close contact. The physical action of regurgitation could be expected to bring some infective material forward into the oral cavity and so allow contamination of the environment. However, van Bekkum *et al.* (1959) have given ample evidence of the non-infectivity of these animals for in-contact susceptibles. In a small experiment carried out at this Institute, four susceptible cattle were housed in close contact with six SAT 3 animals (Table 2, group 3) from the 9th to the 14th week of convalescence. At the end of this period no clinical or serological evidence of transmission of infection was obtained and no virus could be recovered from oesophageal/pharyngeal samples taken on three occasions from the four susceptibles.

Multiplication of virus in an immune animal over a period of several months might be expected to result in some change in the characteristics of the virus both in respect of virulence and of antigenic properties. Preliminary studies with two strains of the SAT 3 virus recovered after 14 weeks in carrier animals have shown that no major changes have occurred. This aspect of the work is continuing to see whether such changes can result from a prolonged carrier state.

SUMMARY

Cattle infected with FMDV strains of different epizootiological origin developed a carrier state which persisted in the majority of animals for several months. Fluid samples taken from the oesophageal/pharyngeal region were assayed for infectivity by plaque counts on BHK monolayer cultures and by mouse inoculation. With one strain of virus, infectivity levels of up to 1000 pfu/ml. were recorded for several weeks after infection but in general the virus content of samples was below 50 pfu/ml.

The sites of virus persistence and multiplication were identified by titration of suspensions of mucosae and epithelia taken *post mortem*. Virus was recovered from 41 of 54 cattle killed 14–196 days after infection. The chief sites of virus multiplication based on the frequency of virus recovery and infectivity titres were the dorsal surface of the soft palate and the pharynx. Virus was recovered less

frequently from the ventral surface of the soft palate and the glosso-epiglottic space and only occasionally from the tonsillar sinuses, tonsils, tongue, trachea and oesophagus. No virus was detected in the turbinates or in the epithelium of the urinary bladder.

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